A seroepidemiological study of canine zoonoses among persons occupationally exposed to dogs

Whitney Sue Krueger

University of Iowa

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A SEROEPIDEMIOLOGICAL STUDY OF CANINE ZOOSES AMONG PERSONS 
OCCUPATIONALLY EXPOSED TO DOGS

by

Whitney Sue Krueger

An Abstract

Of a thesis submitted in partial fulfillment 
of the requirements for the Doctor of 
Philosophy degree in Epidemiology 
in the Graduate College of 
The University of Iowa

December 2011

Thesis Supervisor: Assistant Professor Tara C. Smith
ABSTRACT

Zoonotic diseases continue to emerge and threaten both human and animal health. Overcrowded shelters and breeding kennels create the perfect environment for amplified infectious disease transmission among dogs and present a critical opportunity for zoonotic pathogens to emerge and threaten people who work in close contact with dogs. The objectives of this study were to determine if people with occupational contact with dogs were more likely to have antibodies against *Brucella canis*, canine influenza virus (CIV), and canine respiratory coronavirus (CRCoV) compared to persons with no dog exposure.

A seroepidemiological cohort study was completed, for which 306 canine-exposed and 101 non-canine exposed study subjects enrolled in the study by providing a serum sample and completing a self-administered questionnaire. Evidence of previous exposure was determined by detecting human antibodies against *B. canis*, CIV and CRCoV. Potential risk factors for seropositivity were examined.

Results suggest the overall seroprevalence for *B. canis* antibodies among humans was 3.2%, with 13 subjects testing positive for antibodies against *B. canis* by the rapid slide agglutination test. Several canine-exposed occupations/hobbies not considered in previous studies, including jobs in small breeding kennels and dog show handlers, may be at increased risk for exposure to zoonotic *B. canis*. Occupational risk factors included exposure to a known *B. canis* positive dog (adjusted OR=7.6; 95% CI, 1.01-48.7) and not wearing personal protective equipment (PPE) when caring for a whelping dog (adjusted OR=6.0; 95% CI, 1.02-65.0). In addition, survey results indicated 35% of breeders were not performing *B. canis* testing in their kennels.

Even with indication of antibody cross-reactivity, serological results indicated that the canine-exposed population had higher odds for CIV seropositivity, although the odds were not statistically significant. True human infections with CIV may be occurring at a
low level of incidence, for which this sample size was not large enough to detect a significant difference between the exposure groups. An inverse association between elevated antibodies against CIV with the occupational exposures of examining dogs and wearing PPE when working with dogs are counter-intuitive, but nonetheless it suggests contact with dogs is playing some role in the evidence of antibody production against CIV.

To determine the seroprevalence of antibodies against CRCoV, a competitive enzyme-linked immunosorbent assay (ELISA) was developed to detect human antibodies against CRCoV but control for cross-reacting antibodies against the human coronavirus OC43. All study subjects were negative for antibodies against CRCoV by this competitive ELISA.

Overall, these results suggest that exposure to *B. canis* and CIV is occurring among this study population. Owners and handlers should first ensure the health and safety of the dogs for which they care. To prevent the zoonotic spread of these pathogens to humans, persons in high-risk occupational settings need to be educated of their potential risks, such that they can take proper precautions, including wearing gloves when exposed to a whelping dog and always washing their hands after caring for a sick dog. Lastly, in order to more accurately identify the cross-species spread of these diseases and associated risk factors, prospective studies employing more sensitive serological assays need to be developed. While serological studies have a number of limitations, they are still a valid first tool for identifying emerging zoonotic diseases.

Abstract Approved:__________________________

Thesis Supervisor

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Title and Department

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Date
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by

Whitney Sue Krueger

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Epidemiology in the Graduate College of The University of Iowa

December 2011

Thesis Supervisor: Assistant Professor Tara C. Smith
CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

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has been approved by the Examining Committee
for the thesis requirement for the Doctor of Philosophy
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Shannon Putnam
To my family.

Dedicated to my grandmother, Mary E. Naughtin, for her continued support that made this journey possible.
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ABSTRACT

Zoonotic diseases continue to emerge and threaten both human and animal health. Overcrowded shelters and breeding kennels create the perfect environment for amplified infectious disease transmission among dogs and present a critical opportunity for zoonotic pathogens to emerge and threaten people who work in close contact with dogs. The objectives of this study were to determine if people with occupational contact with dogs were more likely to have antibodies against *Brucella canis*, canine influenza virus (CIV), and canine respiratory coronavirus (CRCoV) compared to persons with no dog exposure.

A seroepidemiological cohort study was completed, for which 306 canine-exposed and 101 non-canine exposed study subjects enrolled in the study by providing a serum sample and completing a self-administered questionnaire. Evidence of previous exposure was determined by detecting human antibodies against *B. canis*, CIV and CRCoV. Potential risk factors for seropositivity were examined.

Results suggest the overall seroprevalence for *B. canis* antibodies among humans was 3.2%, with 13 subjects testing positive for antibodies against *B. canis* by the rapid slide agglutination test. Several canine-exposed occupations/hobbies not considered in previous studies, including jobs in small breeding kennels and dog show handlers, may be at increased risk for exposure to zoonotic *B. canis*. Occupational risk factors included exposure to a known *B. canis* positive dog (adjusted OR=7.6; 95% CI, 1.01-48.7) and not wearing personal protective equipment (PPE) when caring for a whelping dog (adjusted OR=6.0; 95% CI, 1.02-65.0). In addition, survey results indicated 35% of breeders were not performing *B. canis* testing in their kennels.

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To determine the seroprevalence of antibodies against CRCoV, a competitive enzyme-linked immunosorbent assay (ELISA) was developed to detect human antibodies against CRCoV but control for cross-reacting antibodies against the human coronavirus OC43. All study subjects were negative for antibodies against CRCoV by this competitive ELISA.

Overall, these results suggest that exposure to *B. canis* and CIV is occurring among this study population. Owners and handlers should first ensure the health and safety of the dogs for which there care. To prevent the zoonotic spread of these pathogens to humans, persons in high-risk occupational settings need to be educated of their potential risks, such that they can take proper precautions, including wearing gloves when exposed to a whelping dog and always washing their hands after caring for a sick dog. Lastly, in order to more accurately identify the cross-species spread of these diseases and associated risk factors, prospective studies employing more sensitive serological assays need to be developed. While serological studies have a number of limitations, they are still a valid first tool for identifying emerging zoonotic diseases.
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CHAPTER 1
INTRODUCTION

Emerging infectious diseases are diseases whose incidence in humans has increased in the past two decades or threatens to increase in the near future [1]. This includes new infections resulting from the evolution of existing organisms, as is the case with emerging zoonotic diseases that are just now crossing species barriers. Zoonotic pathogens are infectious agents shared among animal and man. It has been estimated that, of the more than 1,400 recognized human pathogens, 64% are zoonotic [2]. Zoonotic pathogens are twice as likely to be emerging or re-emerging, with 73% of the 177 emerging or re-emerging pathogens originating in animals [3]. While viruses and prions comprise only 5% of the catalogue of human pathogens, RNA viruses disproportionately account for 37% of emerging or remerging diseases [2].

Zoonotic diseases continue to emerge and threaten both human and animal health. It is important to monitor the human-animal nexus for emerging pathogens that gain the ability to cross the species barrier. Because these are novel pathogens, humans may have no existing immunity, and there is a potential for an epidemic or even a pandemic to occur. Each year more zoonotic pathogens are classified as emerging threats, and there is a critical need to better understand such pathogen transmission to man [4]. High-risk occupational exposures where workers care for hundreds or even thousands of animals on a daily basis is an important setting for monitoring emerging zoonotic diseases. Because these workers are around such large numbers of animals, if a pathogen were to gain the ability to spread across species, one would expect to first see this among those with such intense animal exposures. Recently, agricultural professionals in routine contact with production animals have been shown to have an increased risk of zoonotic infections and often experience symptomatic illnesses [5-13]. A 2009 review of the literature revealed that veterinarians are often at increased risk for several zoonotic diseases [14]. During a
2003 poultry outbreak of highly pathogenic H7N7 avian influenza virus in the Netherlands, among those exposed to diseased birds, veterinarians had the highest or among the highest attack rates as evidenced by self-reported influenza-like illness symptoms, conjunctivitis, and H7 serology [11]. Five (2.8%) of the 180 veterinarians studied had serologic evidence of exposure, and among the 3,410 persons at risk of H7 infection, the sole death associated with the outbreak was a veterinarian. In addition, veterinarians and veterinary technicians have recently suffered excess morbidity from Hendra virus and monkeypox virus [15, 16]. Recent examples of emerging zoonotic pathogens include the mosquito-borne chikungunya virus [17]; methicillin-resistant *Staphylococcus aureus* reservoirired in swine [18]; Nipah virus and hendra virus, both naturally found in fruit bats [19]; buffalopox virus [20]; and *Streptococcus suis* [21].

When considering that zoonotic diseases continue to threaten human and animal health, and occupational animal exposures provide a valuable setting to monitor pathogen transmission, the canine industry is a worthy but less studied occupational setting where emerging zoonotic pathogens should be monitored. Dogs are a popular companion animal for people worldwide, especially in the United States. There are approximately 74.8 million dogs owned in the United States, with approximately 38% of US households owning at least one dog [22, 23]. The benefits of owning a pet are many, including decreased risks for stress and cardiovascular disease, as well as increased heart attack survival rates and improved psychological and physical well-being. Among children, owning a pet has been associated with reduced risk of asthma and allergies, and improved social skills, self-esteem, and empathy [24]. But this popularity brings consequences. A lucrative dog breeding industry has surfaced, with designer mixed breeds arising as the most recent trend. The approximately 5,000 animal shelters in the United States are growing more crowded, with an average of 6 dogs entering a given shelter every day (Table 1) [25]. These overcrowded shelters and breeding kennels create the perfect environment for amplified infectious disease transmission among dogs. Furthermore,
infectious diseases flourishing among overcrowded dogs present a critical opportunity for zoonotic pathogens to emerge and threaten people who work in close contact with dogs.

In the last decade, two new respiratory RNA viruses, canine respiratory coronavirus (CRCoV) and canine influenza virus (CIV) have emerged, caused widespread outbreaks, and are now considered endemic among dog populations [26-28]. *Brucella canis*, an orally and sexually transmitted bacterium first recognized in 1966, continues to be a problem among large breeding kennels, as no vaccine is available. Testing for *B. canis* is typically not required by state authorities, treatment is difficult, relapse is common, and euthanasia is the only proven method for eradication [29]. Human disease caused by *B. canis* has been documented, but true prevalence rates are unknown. Epidemiological studies examining human infections with these emerging canine pathogens are lacking, as clinical disease is rare or has never before been documented. As CRCoV and CIV are emerging pathogens in dogs, human infections have never been reported or studied; however, other animal-origin influenza and coronaviruses have been shown to cause respiratory disease in humans.

Identifying subclinical and unapparent infections in a highly exposed population will provide a better understanding of cross-species transmission of canine pathogens. An emerging infectious disease first seen in persons with intense canine exposures could indicate an animal pathogen has gained the ability to spread across species. In the case of *B. canis*, a known zoonotic disease, human infection is rarely considered in medical differential diagnoses and detection of a *B. canis* infection is difficult. Hence, clinical cases are likely undiagnosed and untreated [30]. Even more, the general public has a limited knowledge about canine-associated zoonoses. In a 2008 random telephone survey [31] not considering dog ownership, 98% of respondents knew they could get rabies from a dog, however, only 54% knew worms could be transmitted from dogs to humans. Identifying specific disease risks in this occupational group and proper risk
communication would lead to more awareness and consideration of zoonotic diseases by owners and clinical practitioners.

Only a few seroprevalence studies from the 1970s have examined zoonotic *B. canis* transmission [32-34], and none to date have examined zoonotic CIV or CRCoV transmission. To investigate the prevalence of these canine pathogens in humans, a seroepidemiological cohort study was conducted in the United States, predominantly in Iowa and Florida. Evidence of previous exposure was determined by detecting human antibodies against *B. canis*, CIV and CRCoV. Potential risk factors for seropositivity were examined by comparing people occupationally exposed to dogs and non-canine exposed control subjects.

**Specific aims**

**Aim 1.** To determine the seroprevalence of antibodies against *B. canis* among canine-exposed and non-exposed human populations and identify risk factors for evidence of seropositivity.

Hypothesis 1: The seroprevalence of *B. canis* will be higher among canine-exposed persons compared to non-exposed controls, and specific demographic, occupational and behavioral risk factors will be associated with elevated antibody titers (seroprevalence) against *B. canis*.

**Aim 2.** To determine the seroprevalence of antibodies against CIV among canine-exposed and non-exposed human populations and identify risk factors for evidence of seropositivity.

Hypothesis 2: The seroprevalence of CIV infection will be higher among canine-exposed persons compared to non-exposed controls, and specific demographic, occupational and behavioral risk factors will be associated with elevated antibody titers (seroprevalence) against CIV.
Aim 3. To determine the seroprevalence of antibodies against CRCoV among canine-exposed and non-exposed human populations and identify risk factors for evidence of seropositivity.

Hypothesis 3: The seroprevalence of CRCoV infection will be higher among canine-exposed persons compared to non-exposed controls, and specific demographic, occupational and behavioral risk factors will be associated with elevated antibody titers (seroprevalence) against CRCoV.

Objectives

The objective of this cohort study of disease seroprevalence was to determine if people with occupational contact with dogs were more likely to have antibodies against *B. canis*, CIV, and CRCoV compared to persons with no dog exposure. Identifying an at-risk population for canine zoonotic diseases may lead to better disease screening, diagnostics, and treatments for both dogs and humans, the implementation of and adherence to breeding regulations for large kennels, and provide guidance for government and institutional policies to protect persons occupationally exposed to dogs. Chapter 2 provides a review of the three canine pathogens, and the study’s design and research methods are presented in chapter 3. Then the results are summarized in chapter 4 and chapter 5 of this dissertation includes a discussion of the results and future recommendations.
Table 1. Data from The Shelter Statistics Survey, 1994-97

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<tr>
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</tr>
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<tbody>
<tr>
<td>Total number of responding shelters</td>
<td>1,070</td>
<td>1,071</td>
<td>1,012</td>
<td>1,008</td>
</tr>
<tr>
<td>Number of dogs entering responding shelters</td>
<td>2,222,751</td>
<td>2,091,237</td>
<td>2,292,868</td>
<td>2,329,978</td>
</tr>
<tr>
<td>Number of dogs leaving responding shelters</td>
<td>2,090,587</td>
<td>1,888,402</td>
<td>2,117,440</td>
<td>2,117,902</td>
</tr>
</tbody>
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CHAPTER 2
BACKGROUND AND SIGNIFICANCE

*Brucella canis*

First described in 1966, *Brucella canis* is orally and sexually transmitted among dogs, and canine brucellosis typically results in canine abortion and reproductive failures [29]. Since it was first recognized, *B. canis* has been found in several countries, and is most common in Central and South America and the southern region of the United States, particularly rural southeastern regions [35]. Cases have recently been identified in breeding kennels in Japan and China, and sporadic cases have been reported in Europe [36, 37]. In the United States, *B. canis* outbreaks have been documented in Alabama [38], Texas [39], Colorado [40], Illinois and Wisconsin [41, 42], Michigan [43], and Georgia [44-46]. It is estimated that 7-8% of stray dogs in the southern United States are infected [36].

Since *B. canis* is transmitted venereally, canine infections occur most commonly after ingesting placental material, aborted fetuses, or secretions from an infected bitch (female dog) that is either in heat, aborting, or breeding. Canine brucellosis typically presents as abortions in females at 40-60 days of gestation. In stud dogs, epididymitis and scrotal dermatitis are the most common symptoms, which sometimes progress to complete scrotal necrosis [36]. Males shed bacteria in their seminal fluids and urine. The bacteria is typically cleared within a few days of treatment; however re-infection is common and some body fluids may be infectious for weeks as prolonged bacteremia can occur. Infection is most prevalent among wild dog packs, in kennels, and in large-scale breeding facilities where often large numbers of dogs intermingle [35, 47].

*B. canis* pathogenesis

Routes of pathogen entry into a host's body are genital, oronasal, or conjunctive mucosa [48]. *Brucella* gains entry into the body via interactions with a mucosal surface,
where bacteria penetrate the epithelial lining. After entry into the body, bacteria are phagocytized by macrophages and transported to lymphatic and genital organs via the circulatory system, where they reproduce if the host's immune response in the mucosal tissue and lymph nodes fail to eliminate the bacteria [48, 49]. Its intracellular characteristic allows the bacteria to reside in the host’s cells and further evade the humoral immune response. Bacteremia can be prolonged and intermittent. Because *B. canis* is transmitted through vaginal secretions, placenta material, semen, blood, and urine, humans are potentially at risk of infection if they come in direct contact with infected bodily fluids and organs.

*B. canis* infections in humans mimic brucellosis caused by other *Brucella* species [47], and can cause both acute and chronic infections. Acute human brucellosis elicits symptoms similar to that of influenza, which include fever, sweats, headaches, back pains, and physical weakness. Severe infections of the central nervous systems or lining of the heart may occur. Brucellosis can also cause long-lasting or chronic symptoms, including recurrent fevers, joint pain, and fatigue. If untreated, the mortality rate is 2-5% [47].

**Epidemiology of human *B. canis* infections**

Epidemiological data regarding human infection with *B. canis* is lacking, as clinically apparent infections are rare, with approximately 30-40 cases being reported worldwide since the 1960s [35, 36]. Diagnosis of *B. canis* infection in humans is difficult because of an ill-defined clinical presentation of disease, low levels of periodic bacteremia, failure of antibodies to cross-react with antigens used in routine *Brucella* serology, and the selective nature of the organism makes it difficult to grow in culture [50]. Because human *B. canis* infections are rarely considered in differential diagnoses and pathogen identification via serology is difficult, human cases may be underreported [30]. The few reported human cases of *B. canis* chiefly resulted from contact with
aborting bitches (female dogs) or by laboratory exposures to the bacteria; however, the majority of canine infections with *B. canis* do not result in detected human illness.

Veterinarians are usually considered the only at-risk group for *B. canis* infection. In addition to veterinarians, dog breeders and kennel employees are potentially at risk of infection, as they typically come in direct contact with blood, semen, and placentas of dogs.

After the pathogen was identified in dogs, several seroepidemiological studies were conducted in the 1970s to determine if humans were mounting antibody responses to *B. canis*. A 1973 study of US military recruits found a 0.4% seroprevalence of *B. canis* [51]. A 1974 study by Hoff et al. examined 167 animal shelter workers and 43 veterinarians from 21 establishments in 16 counties of Florida [52, 53]. One worker (0.59%) tested positive (agglutinin titers of 1: 200 or greater), but no veterinarians had titers to *B. canis*. In 1975, Monroe et al. examined *B. canis* seroprevalence among a US cohort divided into 4 categories: no exposure (newborn infants), average dog exposure (hospitalized/non-hospitalized patients, hospital employees, and blood donors), high exposure (practicing veterinarians), and people with fevers of undetermined origin [34]. Seroprevalence for the groups were 5.7%, 67.8%, 72.6%, and 80.5% respectively. Seropositivity in newborns was explained by transplacental antibody transfer, similar to reports of puppies born to *B. canis* positive bitches developing antibodies. Due to the overall high seroprevalence of *B. canis*, this study has faced some scrutiny, even though the authors did not believe false positives were confounding their results. Ultimately, this paper suggested a high seroprevalence of *B. canis* antibodies among this population in 1975, with *B. canis* eliciting clinical illness. A serological study in Mexico among hospital patients found a 13.3% seroprevalence (27 out of 203 patients) [54]. Similar to the Monroe study, in 1977 Weber and Brunner examined 1,915 human sera samples (1400 blood donors, 480 clinical patients, and 35 persons exposed to dogs and material infected with *B. canis*) [55]. Three subjects were confirmed positive (0.2%) and all were
healthy blood donors. Using the cross-reacting \textit{B. ovis} antigen, in 1979, Varela-Diaz and Myers found that 23 of 1952 (1\%) of people tested in rural Argentina were positive for \textit{B. canis} antibodies [56]. The last study, in 1982, detected \textit{B. canis} antibodies in 4 (0.3\%) of 1,147 human sera tested; all 4 were patients with undiagnosed febrile illness [57]. Since these seroepidemiological studies, no further research has been done.

Occasional case reports of human clinical infections with \textit{B. canis} are presented in the literature, and most involve direct exposure to a confirmed \textit{B. canis} positive dog [58-63]. The first naturally acquired human infection occurred in 1970, but was the sixth reported human case (the first 4 were laboratory exposures and the fifth was an animal caretaker with asymptomatic serological evidence of exposure) [63]. Another report described a laboratory worker developing a clinical illness after exposure to live M- cells for \textit{B. canis} antigen production [64]. Recently, there have been 2 case reports of \textit{B. canis} causing clinical infections in HIV-positive immuno-compromised patients following exposure to positive dogs [65, 66], as well as the first documented outbreak in humans [67]. A family of 6 (3 adults and 3 children) developed \textit{B. canis} following exposure to a sick bitch and her puppies. Notably, the index case was initially misdiagnosed, and it would have gone undetected if not for the subsequent positive culture. The most commonly reported human symptom of all the human case reports was a recurrent fever of unknown origin.

Public health impact of zoonotic \textit{B. canis}

The dog breeding industry has grown significantly since \textit{B. canis} was first recognized in 1966 and the first serologic studies were performed in the 1970s. \textit{B. canis} has become a major source of economic loss in both large and small US dog breeding facilities, as a single outbreak can lead to the euthanasia of hundreds of dogs [29]. No vaccine is available and treatment is not recommended as antibiotics are costly and rarely cure infection [47]. Canine brucellosis is difficult to treat because its intracellular
characteristic allows the bacteria to reside in the host’s cells and evade the host’s immune response. If prevention strategies fail, infected dogs must be eliminated from the kennel to control an outbreak [29]. Pet stores do not routinely test for *B. canis*. In the United States, testing regulations vary widely by state, and often government regulations do not require routine testing by dog breeders and kennel owners. Because the commercial dog breeding industry has sparse regulatory requirements, owners and employees often lack professional training in animal health, which can lead to inadequate hygiene and infection control practices. Kennels may also be lax about disease testing and general veterinary intervention. Each year hundreds of thousands of puppies are born and sold to pet stores, other commercial breeders, unknown parties, and even overseas, by means of improper sales and breeding practices. These transactions do not require *Brucella* testing; consequently, it is in these facilities where *Brucella* outbreaks are most likely to occur as dogs and people are readily exposed to this zoonotic pathogen.

**Influenza A viruses**

The three species, or types, of influenza viruses (A, B, and C) are genus members of the family Orthomyxoviridae. Humans can be infected with all types, but influenza A is the most virulent human influenza pathogen. Wild aquatic birds are the natural reservoirs for most influenza A viruses, but through various modes of transmission and evolution, influenza A viruses can also infect domestic birds, and a wide range of mammals, including humans, pigs, horses, dogs, cats, and marine mammals. Influenza A viruses are further classified by subtype (serotype), which is based on two main surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). Sixteen HA and nine NA glycoproteins are recognized. Subtypes of influenza A currently circulating among people worldwide are H1N1, H1N2, and H3N2 viruses. Influenza A subtypes are further classified by strain.
Influenza virus evolution

Influenza viruses evolve by three distinctive mechanisms [68]. Antigenic drift creates new strains within an influenza subtype due to minor, unpredictable point mutations in the genes that produce the HA and NA surface proteins. These new influenza strains may not be recognized by humans’ or animals’ immune systems (both from previous infections or vaccines); therefore, susceptible species can be infected with multiple strains of the same influenza subtype. Antigenic drift explains why seasonal flu vaccines must be updated annually.

The other type of genetic change is called “antigenic shift”, which can occur by two processes. First, an abrupt, major change of an influenza virus can lead to direct species-to-species transmission (such as avian-to-pig transmission, or pig-to-human transmission). This is a notably rare event for any influenza virus. Antigenic shift can also occur via the mixing of two or more influenza viruses (e.g. a human and animal influenza A virus) in an intermediate host (e.g. pigs) to create a new influenza subtype (new HA or NA). This exchange of genetic material between multiple influenza viruses is called “reassortment”. In both instances of antigenic shift, people and animals may have no immunity to this new virus and thus the virus is able to spread rapidly through a population and potentially lead to a pandemic.

Influenza pandemics occur when humans have little or no immunity against a novel influenza virus subtype that transmits efficiently from human to human. Often the human immune system is naive to such pathogens, because the viruses present with antigens of animal virus origin. Whereas the pandemics of 1918, 1957, and 1968 had genetic components from avian viruses, pandemic viruses may arise from any number of animal influenza viruses, such as the case with the recent pandemic H1N1 (2009) virus. This novel H1N1 pandemic virus had a lineage of human, swine, and avian origins. Hence, exposure to animal species harboring influenza A viruses may play a role in the origination or spread of a novel virus.
H3N8 canine influenza virus

In the case of canine H3N8 influenza A virus, a rare antigenic shift occurred in the equine H3N8 virus, which allowed for direct horse-to-dog transmission without viral reassortment [28]. The new canine virus was first isolated in Florida in 2004 from racing greyhounds experiencing respiratory disease, although it is believed that the actual event of viral transmission between racing greyhounds and racing horses had occurred several years before [28, 69]. Up until this direct transmission, dogs were only known to be infected with human influenza viruses and subsequent dog-to-dog transmission of influenza viruses was not thought to occur [27]. This new virus had a novel genetic makeup and was efficient in spreading dog-to-dog; hence, the new H3N8 canine influenza virus (CIV) was classified.

After CIV was first discovered, many retrospective and cross-sectional serological studies were carried out in US dog racing populations. Between 2004-2006, CIV outbreaks affected thousands of greyhounds associated with race tracks in nine states [27]. CIV was first identified in the general pet population 8 months after it was first discovered in the racing community [28, 70]. CIV has been confirmed in at least 30 US states, and it is considered an enzootic pathogen in Florida, New York, Philadelphia, and Colorado, with additional case clusters in New Jersey and Wyoming [28, 69, 71]. As with other influenza viruses, its evolution is unpredictable and viral changes must be continually monitored as it moves through the dog population [70].

In dogs, CIV presents as respiratory symptoms similar to “kennel cough”; however, CIV has a higher attack rate (60-80%) due to their naive immune systems’ susceptibility to this new virus. In May 2009, the USDA approved an influenza vaccine for dogs [72]. The killed-virus vaccine proved to decrease the spread, signs, and severity of CIV infection. Because dogs have no existing immunity to the virus, the USDA has recommended that dogs who are 1) vaccinated for Bordetella, 2) entering into or adopted
from shelters, or 3) boarded in kennels or visit doggie daycares or other canine social settings should be vaccinated.

**Influenza virus pathogenesis**

Influenza viruses are transmitted through inhalation of infected aerosolized droplets. After inhalation, influenza virus binds to respiratory tract cells, with the aid of hemagglutinin surface glycoproteins. Upon entry, the virus converts RNA to mRNA. Then with the aid of the neuraminidase surface glycoprotein, newly formed viral particles bud from the host wall via a proton channel formed by the M2 protein. The budding process damages the epithelium and elicits an inflammatory response by the host.

Influenza virus pathogenicity and virulence is influenced by both host and viral factors [73]. Viral mutations often influence viral pathogenicity, for example, a D222G mutation in the HA protein provided the 2009 pandemic H1N1 influenza A virus the ability to bind to α2,3 sialylated glycans, leading to infection of human ciliated bronchial epithelial cells [74]. The A143T mutation in the H7N7 avian influenza virus increased attachment to bronchial epithelial cells and alveolar macrophages in humans [75]. Other viral factors that affect pathogenicity and virulence include the virus's ability to bind to and bud from host cells, restricting cytopathogenic effects, and escaping detection by the host's immune system via antigenic variation or recombination with other influenza virus strains. Host factors that affect pathogenicity and virulence include the presence of target cell receptors and enzymes, immunocompetence, existing antigenic immunity, and the ability to control viral replication by its inflammatory response without damaging the host.

**H3N8 CIV in humans**

It is assumed that CIV is incapable of infecting humans, but to date, no studies have been conducted on humans. Humans have, however, been shown to mount immune responses against influenza viruses from birds, pigs, and horses. As canine cases have been linked to dog racetracks, kennels, and shelters [76], where a large number of dogs
are housed together, employees working at these facilities are potentially exposed to CIV and at risk of acquiring a zoonotic infection. If CIV gains the ability to infect humans and spread human-to-human via viral mutations or further reassortment, there will be potential for a large human epidemic to occur.

Coronaviruses

Coronaviruses (CoVs) are large, single-stranded RNA viruses classified into 3 antigenic groups, although taxonomy may soon be revised [77]. They account for respiratory, enteric, and/or systemic disease in both mammals and birds. Group 1 CoVs include canine, feline, swine, porcine, and human coronaviruses. Group 2 CoVs are classified as “bovine-like” (subgroup 2a) and “SARS-like” (subgroup 2b) viruses. Members of group 2a include bovine, mouse, porcine, human, equine, and canine coronaviruses. CRCoV is a Group 2a coronavirus because of the distinguishing presence of the hemagglutinin-esterase (HE) surface glycoprotein in addition to the viral spike gene. Group 2a contains both human and animal pathogens; however, CRCoV possesses different biological and genetic properties compared to the Group 1 enteric canine coronavirus. CoVs newly isolated from bats and wild carnivores have been added to Group 2b. Group 3 includes CoVs of avian origin. The development of canine CoVs followed the classic model of coronavirus evolution, based upon accumulations of point mutations, insertions, and deletions within the viral genome [77].

Canine infectious respiratory disease

Canine infectious respiratory disease (CIRD), or “kennel cough” is a highly contagious multifactorial disease typically found in rescue centers, boarding kennels, and training kennels, where dogs are housed in groups. Typical symptoms present as a mild cough and a rapid recovery occurs after 1-3 weeks. In some instances, however, a fatal bronchopneumonia can develop [78]. As CIRD has a multifactorial etiology, several viruses and bacteria have been identified as primary pathogens or causes of secondary
infections [79]. However, widespread use of vaccines against known causative agents has not reduced the incidence of CIRD; it is still a problem in many kennels. The morbidity and mortality caused by CIRD not only directly affect the dogs, but the costs of treatment, welfare repercussions, and delays in re-homing and training, place a large economic burden on these kennels and centers [26, 78].

Canine respiratory coronavirus

As the pathogenesis of “kennel cough” had not been thoroughly investigated since the 1970s and the current vaccine proved ineffective in preventing CIRD, researchers in the United Kingdom sought to identify additional etiologic agents for CIRD [78]. They analyzed respiratory samples from diseased dogs living in rescue centers and detected a novel coronavirus, dubbed canine respiratory coronavirus (CRCoV), which was implicated to play a role in the CIRD complex [79]. Using a nested PCR, CRCoV was detected in 32/119 tracheal and 20/119 lung canine samples, and dogs with mild clinical symptoms were more likely positive, although statistical significance was not reported [78]. The seroprevalence of antibodies against CRCoV was 30.1% among 111 dogs on the first day of entry into a rehoming kennel and 99% after 21 days, which indicated CRCoV was a highly contagious virus. It remains unclear if CRCoV either predisposes dogs to further, more serious infections, or functions as a primary pathogen for infection [26].

Following its discovery in the United Kingdom, evidence of CRCoV infection or seropositivity has been found among dogs of various clinical histories in Japan, Italy, New Zealand, Korea, Canada and the United States. Analyzing respiratory swabs samples collected from 96 Japanese dogs with respiratory and/or enteric signs of illness, researchers found two dogs to be positive for CRCoV infection by reverse transcription PCR in 2005 [80]. Another study conducted in Japan examined 898 canine serum samples collected between 1998-2004 with unknown clinical histories were analyzed for
antibodies against CRCoV [81]. Antibodies were detected in 160 (17.8%) of the samples, including samples collected in 1998. A third Japanese study examined the seroprevalence of CRCoV among various Japanese dog populations [82]. Sera from 373 clinically healthy dogs collected in 2004, sera from 90 dogs with respiratory signs and 37 dogs with diarrhea collected in 2004, and sera from 225 seemingly healthy dogs collected in 1994, were all examined for evidence of antibodies against CRCoV. Results indicated seropositivity among 24.9% of the 2004 dogs and 23.1% of the 1994 dogs. Dogs with respiratory signs were significantly more likely to be seropositive than the healthy dogs (P<0.0001) and the dogs with diarrhea (P<0.05).

In Italy, sera from 216 dogs of various clinical histories collected from 1994-2006 were tested for antibodies against CRCoV [83]. Antibodies were only detected among sera collected in 2005 (38.3% seroprevalence) and 2006 (26.8% seroprevalence), indicating the virus was not circulating among Italian dogs prior to 2005. In addition, the authors found a higher seroprevalence in dogs >6 months of age (51.6%) compared to younger dogs (26.0%), although statistical significance was not reported. In a 2009 study conducted in New Zealand, 251 dogs of varying clinical histories were tested for antibodies against CRCoV, for which 73 (29%) sera were seropositive [84]. The authors also found that older dogs were more likely to be seropositive for CRCoV antibodies, although statistical significance was not reported. In a 2010 Korean study, respiratory samples from 69 euthanized dogs and 40 dogs which had died of natural causes, all of whom presented with either subclinical symptoms, skin disease, vomiting, or respiratory disease were tested for molecular evidence of CRCoV infection [85]. Three dogs (2.8%) were found to be CRCoV positive, and all 3 dogs were reportedly ill at the time of sample collection.

A 2006 serological study examined banked canine serum samples collected from dogs with unknown clinical histories in the United Kingdom, United States, Canada, Ireland, and Greece [26]. Serological results indicated 297 (36.0%) of 824 of dogs from
the United Kingdom had antibodies against CRCoV, including dogs from England, Scotland, Wales, Ireland, and Greece. In addition, 521 (54.5%) of 956 US dogs and 26 (59.1%) of 44 Canadian dogs were seropositive for CRCoV antibodies. Sera from 32 US states were tested. In Iowa, 2 out of 5 dogs (40%) tested seropositive for CRCoV. Seroprevalence was 57.7% (75/130), 66.7% (14/21), 60.0% (6/10), and 40.9% (18/44) in Wisconsin, Illinois, Indiana, and Florida, respectively.

Coronavirus evolution

It is believed that human coronavirus strain OC43, a cause of the common cold, emerged as result of a transpecies infection caused by bovine coronavirus, as they share significant antigenic and genetic similarities [86]. This may be true for other Group 2a “bovine-like” coronaviruses as well [87], as CRCoV shares a high amino acid similarity to bovine CoV and human CoV OC43 (approximately 96% identity with the bovine spike gene) [79]. Therefore, CRCoV was most likely transmitted from cattle to dogs [88], and OC43 similarly transmitted from cattle to people [86].

Like other RNA viruses, coronaviruses mutate at a high frequency because their RNA dependent RNA polymerase is inherently error-prone leading to point mutations and even deletions [89]. Relatively minor genetic changes can provide new coronaviruses with the ability to efficiently spread human to human, by gaining the capability to either bind to a new receptor or replicate in cells of a new host [90]. Coronaviruses also have a high RNA recombination frequency (as high as 25%), which is unusual for unsegmented viruses, but common in influenza’s segmented genome [91-93]. This occurs during RNA synthesis, when discontinuous transcription and polymerase jumping can occur, causing the polymerase to dissociate from its template at random and switch to a homologous site on a different RNA template. From there, RNA synthesis is completed [89].
Coronavirus replication and pathogenesis

Coronaviruses maintain a broad host range, including many mammalian and avian species. Variations in host range specificity, tissue tropism, and pathogenesis are attributed to the spike glycoprotein [94]. Coronaviruses are spread through various modes of transmission; CRCoV is transmitted through inhalation of infected aerosolized droplets. After entry into the body, coronaviruses attach to specific cellular receptors via the spike protein [95]. Viral and cell membranes fuse and the nucleocapsid is released into the cell. After entry, viral RNA and proteins are synthesized in the cytoplasm. New virions assemble via budding into intracellular membranes and are then released through cell secretory mechanisms.

The pathogenesis of CRCoV is not yet clear. In dogs, it is assumed that CRCoV elicits only a subclinical or asymptomatic disease, but that damage to the respiratory epithelium during viral replication may lead to clinical secondary infections by other respiratory pathogens [96]. If the CRCoV spike glycoprotein was to gain affinity for the human respiratory epithelial cell receptor that OC43 employs (Neu5,9Ac2-containing moiety) [95], CRCoV could potentially replicate in human cells and cause human infections.

CRCoV in humans

Human coronaviruses (HCoVs) such as the Group 2a strain OC43 are thought to cause 15-30% of common colds [97], but specific examination of CRCoV infection in humans has not been conducted. As respiratory infections are exacerbated by overcrowding, canine CRCoV cases have been linked to shelters, boarding kennels, and doggie day care centers, where a large number of dogs are present; therefore, employees working at these facilities are at potential risk for a zoonotic infection with CRCoV. The emergence of new HCoVs from animals, including the severe acute respiratory syndrome coronavirus, proved that HCoVs could be of zoonotic origin, highly pathogenic, and
cause severe upper and lower respiratory tract infections. No antiviral drugs are available to prevent or treat HCoV infections.

Public health impact of zoonotic CoV infections

Coronaviruses cause respiratory disease in many species, and four strains are known to infect humans and cause a significant percentage of all common colds. In 2003, however, a new coronavirus emerged in Asia that was much more virulent than the common cold. These severe acute respiratory syndrome (SARS) illnesses yielded the SARS-associated CoV as the causative agent. The virus spread to more than two dozen countries in North America, South America, Europe, and Asia and caused more than 8,000 human infections and more than 700 deaths. Upon investigation into its origins, SARS-CoV was determined to be a zoonotic pathogen that likely evolved to infect humans by a sequence of transmission events between humans, market animals for sale in China, and bats [98]. With bats believed to be the viral reservoir [99], the palm civet cat served as the intermediate viral host, and human interactions with the cats at food markets (where sanitary conditions were poor) provided an interface for viral transmission from animal to human and the evolution of the epidemic SARS-CoV human strain [98]. The SARS epidemic renewed concern for animal coronaviruses to serve as agents of direct and indirect zoonoses [77, 87].

Coronaviruses must continually be monitored as their widespread prevalence, extensive host range, various disease manifestations, and increased frequency of recombination events all underline their potential for interspecies transmission [99].

Determining causation

An infection is defined as the colonization of a host organism by a pathogen, in which replication of the microorganism occurs. When establishing a causal relationship between a pathogen and a clinical disease, Koch's postulates are often applied: 1) the microorganism must be present in every case of the disease, 2) the microorganism must
be isolated from a diseased host and grown in pure culture, 3) the cultured microorganism should induce disease in an experimental host, and 4) the microorganism must be re-isolated from the inoculated host and be identical to the original causative agent [100]. While these postulates have limitations, especially when the microorganism cannot be grown in pure culture or when no animal model exists, Koch's postulates serve as a practical guideline for ascertaining cause and effect between an infectious agent and a disease. However, when a pathogen produces a subclinical infection, or when a non-virulent microorganism elicits clinical disease in an immunocompromised patient, Koch's postulates are not an effective tool for determining causality. This epidemiological cohort study of disease seroprevalence aimed to determine if humans had been previously exposed to canine pathogens by detecting antibodies directed against the pathogens in question. Because of its retrospective nature and disease diagnosis via serology at a single time point, this study design did not allow for the ability to identify true infections and consequential clinical disease. Alternatively, Bradford-Hill's criteria for causation is an epidemiological tool for determining a causal relationship between cause and effect [101-103]. These nine criteria are: strength of association, consistency, specificity, temporality, biological gradient, plausibility, coherence, reversibility, and analogy. These criteria can be considered when determining whether specific exposures were associated with an elevated antibody response. For example, odds ratios can examine the strength of association between an exposure and outcome. Because this study included serological sampling at a single time point, it alone cannot confirm infection has occurred. If however, if is found that specific exposures suggest a possible association between a causative etiological agent and the outcome, future prospective studies would be warranted to identify expected seroconversions. For confirmation of an infection via serology, it is imperative to have acute and convalescent sera samples. However, in instances where the outcome is rare and clinical disease unlikely, to capture acute cases of subclinical illness would be very difficult. Seroprevalence studies are the best
epidemiological approach when first examining the potential for rare and seemingly unapparent infections in a population.

**Significance**

Only a few seroprevalence studies from the 1970s have examined zoonotic *B. canis* transmission[32-34], and none to date have examined zoonotic CIV or CRCoV transmission. This study will focus on several occupational groups readily exposed to many dogs and therefore, expected to be the primary population most likely affected by these pathogens. The dog breeding industry has grown dramatically in recent years and this will be the first modern study to examine US human populations at highest risk for emerging zoonotic canine infections. This study could advance human diagnostics for animal pathogens, demonstrate the critical need for instituting policy measures to better mandate disease testing and hygiene control practices in kennels and shelters, as well bring awareness to those occupationally exposed to dogs, particularly sick dogs, of their potential risks.
CHAPTER 3
RESEARCH DESIGN AND METHODS

Overview of study design

A seroepidemiological cohort study was conducted to meet the study aims. The study was approved by the University of Iowa and the University of Florida's institutional review boards. The target population included breeders, kennel employees, veterinary personnel, animal shelter workers, greyhound racetrack employees, and dog show handlers whose work or hobby involved exposure to multiple dogs. A non-exposed control group consisted of individuals who had not been exposed to multiple dogs as part of their work or hobby nor had pet dogs in their household in the last 5 years. All participants had to be at least 18 years of age and self-report no current immunocompromising conditions.

Recruitments were based on a convenience sample of the target population primarily from Iowa and Florida. Breeders, kennels, shelters, and veterinary clinics were identified through state databases of licensed breeders and practicing veterinarians. Local shelters, clinics, and greyhound racetracks were also identified through internet searches. Organizations and staff were invited to participate in the study via a mailed letter with a telephone call follow-up. Enrollments typically occurred at the participants' place of employment. Recruitment also occurred at large public venues including dog shows, agility trials, and trade shows. After obtaining approval from the venue organizers, a small table was set up where potential participants could ask questions and enroll in the study. Non-exposed controls were faculty, staff, and students from the University of Iowa and the University of Florida. After informed consent was obtained, participants completed a self-administered questionnaire and permitted collection of a blood specimen.
Sample size

As zoonotic disease transmission from dogs to man has not been well studied and prevalences were expected to vary between pathogens, power calculations and sample size were difficult to estimate *a priori*. Hypothesizing low prevalences, an exposed to non-exposed ratio of 3:1 was selected. This study aimed to enroll 300 exposed and 100 non-exposed subjects in a seroepidemiological cohort study of canine zoonoses among people occupationally exposed to dogs compared to people not exposed to dogs. The sample size estimate necessary to have 80% power to detect various true unadjusted differences in prevalence between groups would be 300/100 when the true prevalence was 12.5% and 3% (Table 2).

Participant recruitment

Recruitments were based upon enrolling convenience samples of the target population. Advertisements, posters, emails, letters, and existing databases were employed to recruit subjects. Dog breeders with more than 3 breeding males or females are required by law to be licensed. As these lists are available to the public, they were used to find breeders within Iowa and Florida. Databases of licensed practicing veterinarians were also used. Hobby breeders with less than 3 breeding females and less than $500 gross income from selling dogs are not required to be licensed. To find these “Mom and Pop” breeders, local advertisements for puppy sales were monitored.

To reach smaller, private entities such as small-scale breeders, veterinary clinics, kennels, and shelters, the investigator first sent a letter to the potential subject/business owner that described the study. As an opt-out procedure, the letter included the principle investigator's phone number and email address and instructions to notify her if they chose not to be contacted in the future. For those who did not choose to opt-out, approximately 7 days after sending the introduction letter, research team members called the potential subject. Within one week, calls were made in the morning, afternoon, evening, and
weekend. At the end of 3 weeks, if the potential subject/business owner had not been reached, they were excluded as a potential subject and removed from the contact list. Once a potential subject was reached by phone, a phone script was followed by the researcher. The phone script provided information about the study, asked questions to verify eligibility of the subject, and determined a time for 1-2 research members to visit the facility for enrollment.

In order to recruit employees at dog race tracks, a liaison such as a state or track veterinarian was first contacted and asked if he or she would facilitate contact with the racing directors who ultimately granted permission to visit the track and recruit employees.

To reach potential participants at large venues such as dog shows and trade shows, the principle investigator first contacted show organizers to introduce the study and obtain a "letter of agreement" for IRB purposes, which permitted research personnel to target participants and spectators for study recruitment and enrollment during the shows. When recruiting at a large venue, a small table was set up where potential participants could ask questions and enroll. A 2x3 ft poster was displayed to catch people’s attention in addition to announcements sometimes broadcasted over the public address system. The research team spoke directly to the potential subjects to explain the purpose of the study. Once determined that the inclusion criteria were met, the willing participants were enrolled in the study at the venue location.

Non-dog exposed controls were enrolled from a pool of University of Iowa College of Public Health faculty, staff, and students as well as University of Florida College of Public Health and Health Professions faculty, staff, and students. An email advertisement was sent to all associates, inviting them to come to designated meeting times to enroll in the study if eligible. From there, recruitment of controls mimicked that for recruiting exposed subjects at large venues: a small table with a poster was set up, where potential participants could ask questions and enroll. The research team spoke
directly to the potential subjects to explain the purpose of the study. Once determined that
the inclusion criteria were met, the willing participants were enrolled in the study.

**Enrollment**

At a single encounter, background data, animal exposure, and related risk factors
of study subjects were collected using a self-administered questionnaire specifically
developed for this study (see Appendix A). A blood sample was also collected from
every participant to verify presence of antibodies against *B. canis*, CIV, and CRCoV.

Per the Institutional Review Board (IRB) guidelines, participants were first
presented with the study's IRB-approved Informed Consent Form. Immediately after
informed consent was obtained, subjects were asked to complete a 10 minute self-
administered questionnaire. Then approximately 5ml of blood were collected via
venipuncture. All participants were given a free t-shirt for participating, as well as their
serological result of the *B. canis* test if they consented to receive it. In the event
venipuncture was unsuccessful and blood could not be collected, the subject still received
a t-shirt and was considered enrolled in the study for IRB purposes; however, no further
study procedures were performed, including questionnaire data analyses. Blood could
not be obtained from 24 (6%) of the participants (12 canine-exposed and 12 non-
exposed).

**Measuring exposure and other covariates**

The cut off qualification for canine exposure was set at 5 years. For *B. canis*,
dogs can remain culture positive for years, and seropositivity can last 6-12 months [48].
In the report of the first naturally acquired human case of *B. canis*, the woman sustained a
low seropositivity one year after completion of therapy [63]. In another published case
report, the authors noted that 8 months after the case’s symptoms, serology continued to
be positive [104]. The case was never again tested. Persistence of IgG is presumably
associated with persistence of clinical disease (seen for 6-11 months), while IgM may
remain for years in persons with subclinical infections [105]. In addition, antibodies against the cell wall of *B. canis* generally last only during bacteremia, while antibodies against cytoplasmic antigens persist longer (6-12 months) [106]. For influenza viruses, there are conflicting reports of when antibodies wane, but most agree it can last for years in humans [107]. The same is true for coronaviruses. Consequently, the investigators set an estimate of 5 years to appropriately classify subjects are exposed or unexposed. This seemed a sensible midpoint so as to minimize misclassification among exposed as unexposed or vice versa.

The exposed population included any individual occupationally exposed to multiple dogs in the last five years. Inclusion criteria for participation included 1) close contact (~3 feet) with multiple dogs as part of work or hobby within the last 5 years, 2) at least 18 years old, and 3) no current, self-reported immunocompromising conditions. Various occupations were targeted for recruitment, including breeders, kennel and shelter workers, veterinarians and veterinary staff, race track employees, and dog show enthusiasts. Persons merely owning pet dogs were not a target for this study. As isolated pet dogs are not at high risk for these canine infections, the average pet owner was unlikely to be exposed to these canine pathogens of interest.

The control population included any individual who had not been occupationally exposed to multiple dogs and had no pet dog in his/her household in the last five years. Inclusion criteria for participation included 1) no close contact (~3 feet) with multiple dogs as part of work or hobby, 2) no pet dogs in household in the last 5 years, 3) at least 18 years old, 4) no current, self-reported immunocompromising conditions.

Questionnaire

Subjects were asked to complete a 10 minute self-administered questionnaire (see Appendix A) that collected demographics; general lifetime exposure data for various animals, including pet dogs; a brief work history (including occupational exposures to
dogs); and medical information that may affect whether or not they are more likely to
develop an illness or infection (such as chronic diseases). Dog exposure questions asked
for how long subjects had worked with dogs and to how many dogs on average they
tended (to ascertain dog-years of exposure). Exposure to specific dog groups (e.g.
breeding female, spayed female, non-breeding male) was also assessed. Questions asked
about personal protection practices when around dogs, and in particular, when exposed to
sick dogs or whelping (birthing) females. Data was collected on any known canine
infections with *B. canis*, CIV, and CRCoV, as it was expected that veterinarians and
some breeders would know if their dogs had been infected with these specific pathogens.
Vaccine data for the human influenza vaccine as well as any accidental animal vaccine
exposures were collected as well, in an attempt to control for cross-reactivity in the
serologic assays.

Exposure to animals that are also affected by brucellosis, including cattle, sheep,
pigs, goats, deer, and elk was documented such that potential cross-reactivity between
antibodies against other *Brucella* species (*B. abortus*, *B. melitensis*, *B. ovis* and *B. suis*)
with the serology assay could be examined, and if necessary controlled for in statistical
analyses.

Measuring outcome

Study outcome was detection of *B. canis*, CIV, or CRCoV antibodies via
serological assays. However, the serological assays developed for this study have their
limitations. A “positive” result could indicate 1) the subject was exposed to the pathogen
in question sometime in the past; clinical infection may or may not have been present, or
2) antibodies to other pathogens cross-reacted with the antigen in the assay to produce a
false positive result. A “negative” result could indicate 1) the subject was not exposed to
the pathogen at a level sufficient to produce an immune response, or 2) existing
antibodies did not react with the antigen in the assay. Cross-reactivity was controlled, to
an extent, by 1) running a confirmatory serological assay and validation work, when possible, 2) testing sera for suspected possible cross-reacting antibodies and including vaccination history in statistical analyses, as was done for CIV, and 3) by running competitive assays that blocked antibodies reacting to homologous antigens, as in the case with CRCoV.

**Laboratory methods**

Whole blood specimens were transported on ice to the laboratory within a few hours of collection. Blood tubes were spun at 3000xg for 15 minutes at room temperature to separate serum. All collected serum was aliquoted and frozen at -80°C.

**Brucella canis**

Routine brucellosis diagnosis does not include a test for *B. canis*, but it has been reported that the rapid slide agglutination test (RSAT) could be a suitable screening test for identifying human antibodies against *B. canis* [108-111]. For this study, sera were tested for antibodies against *B. canis* by adapting a commercial canine brucellosis RSAT kit used to screen dogs for *B. canis* infections.

**Rapid slide agglutination test.**

The D-Tec CB test kit was commercially available from Synbiotics Corporation (Kansas City, Missouri) (Figure 1). A suspension of whole *B. ovis* stained with Rose Bengal cross-reacts with IgM and IgG antibodies against *B. canis*. To decrease the possibility of false positives, the kit includes a 2-Mercaptoethanol (2ME-RSAT) confirmatory procedure that was used with positive reactors to the initial RSAT screen to remove common non-specific agglutins in sera, including cross-reacting IgM against other bacteria. The kit instructions were mostly followed, save for a few modifications. For the initial screen, equal parts (60μl) of serum and antigen were mixed within an oval circle on a card with a stir stick. The card was rocked for 3-5 minutes to swirl the mixture
and then observed for agglutination (Figure 1). If positive, the 2ME-RSAT procedure was then completed. First, 2ME and serum were mixed in equal parts (60μl) in a test tube. This mixture was then added to the antigen in equal parts (60μl) and the subsequent RSAT steps followed. Traditionally, RSAT is considered highly sensitive but less specific; however, one report found the test’s specificity to be higher [35]. In this case, the RSAT sensitivity/specificity was 70.58% and 83.34%, respectively, and the 2ME-RSAT sensitivity/specificity was 31.76% and 100%, respectively [112].

Influenza

The microneutralization (MN) assay and the neuraminidase inhibition (NI) assay were used to test for antibodies against the H3N8 canine influenza virus, and the hemagglutination inhibition (HI) assay to test for cross-reacting antibodies against the human H3N2 influenza virus. The canine influenza virus isolate A/Canine/Iowa/13628/2005 (H3N8) was kindly provided by Dr. Kyoung-Jin Yoon at Iowa State University, Ames, IA. Canine sera from dogs confirmed positive for CIV were kindly provided by Dr. Cynda Crawford at the University of Florida, Gainesville, FL.

Virus culturing

Canine and human influenza viruses were grown in 10-day old embryonated chicken eggs (Charles River Laboratories, Wilmington, MA). Following injection of 100μl of virus diluted 1:10 in Dulbecco's Phosphate Buffered Saline (PBS) into the allantoic cavity, eggs incubated for 72hrs at 37°C and were then chilled to 4°C for 4hrs. Allantoic and amniotic fluids were harvested from each egg and screened for virus by the hemagglutination assay (HA). Fluids with the same HA titer were pooled, aliquoted, and frozen at -80°C.

Because after 4 egg passages a high viral titer for the H3N8 canine influenza could not be achieved with egg culturing alone, virus was then propagated in Madin-
Darby Canine Kidney (MDCK) cells. Cells were seeded in 150cm² cell culture flasks (Corning, Corning, NY), and upon 80-90% confluency, flasks were washed 3 times with 1X Minimum Essential Media with Earles salts and L-glutamine (MEM) (Gibco®/Invitrogen, Carlsbad, CA); the MEM remained on the cells for 10min during the final rinse. Residual MEM was removed with a pipette. One milliliter of p4 CIV diluted 1:4 in Olsen's infection media (1X MEM; 100 mg/ml streptomycin and 100,000 IU penicillin (Fisher Scientific); and 0.01mg/mL Tosyl Phenylalanyl Chloromethyl Ketone (TPCK) treated trypsin [Worthington Biochemical Corporation, Lakewood, NJ]) was inoculated onto each flask. Cells were incubated with rocking for 4hrs at 36°C with 5% CO₂. An additional 15ml of Olsen's media was then added and cells were incubated at 36°C with 5% CO₂ until 70-90% CPE was visualized (3-5 days). A mock-infected flask was included as a negative control. Cell supernatant was harvested and tested by HA to determine the HA titer.

Hemagglutination assay

Turkey or guinea pig red blood cells in Alsever's solution (Lampire Biological Laboratories, Pipersville, PA) were washed 3 times to remove Alsever's solution. On a 96-well V-bottom microtiter plate (Greiner Bio-One, Monroe, NC), viral specimens were serially diluted in PBS (50μl per well). A 0.5% turkey (for canine influenza virus) or 0.65% guinea pig (for human influenza virus) red blood cell (RBC) suspension in PBS (50μl per well) was added to the dilutions. After 1hr at 4°C (canine influenza virus) or room temperature (human influenza virus), plates were examined for the presence or absence of RBC settling. The highest dilution of the virus with complete hemagglutination was recorded as the HA titration end point. The HA titer per 50μl was the reciprocal of the end point; HA units per milliliter was the reciprocal of the end point times 20.
Hemagglutination inhibition (HI) assay:

A quantitative HI assay was used to examine subjects' sera for antibodies with hemagglutinin (H3) subtype specificity. Following previous reports, human sera was tested by using the Centers for Disease Control and Prevention (CDC) HI assay protocol against an H3N2 human influenza A virus (A/Brisbane/10/2007 [CDC]) [7, 8]. Sera were pretreated with receptor-destroying enzyme (RDE) (Denka Seiken, Tokyo, Japan), heat inactivated at 56°C for 30 minutes, and then hemabsorbed with guinea pig erythrocytes to remove nonspecific hemagglutination inhibitors and nonspecific agglutinins. Using a V-bottom microtiter plate, 25μl of sera diluted 1:10 in PBS was serially diluted down each column. Four HA units of virus (25μl) were added and the plate incubated for 15 minutes at room temperature. Each serum sample included a negative control well with no virus, and assay controls of positive H3 antisera and uninfected sheep serum from a recent World Health Organization Influenza Reagent Kit were also included. A 0.65% suspension of guinea pig erythrocytes was then added to all wells and the plate incubated at room temperature for 1hr. If specific antibodies were present, they neutralized the influenza virus antigen's hemagglutinating property. The plates were manually read to determine the highest titer at which hemagglutination was inhibited. Sera were considered positive at an HI titer ≥1:40.

Microneutralization (MN) assay

Following previous reports [5, 13, 113-117], an MN assay adapted from that of Rowe[118] was used to detect hemagglutinin (HA)-specific antibodies that neutralize the influenza virus's ability to infect MDCK cells. Sera were heat inactivated at 56°C for 30 minutes and screened at a 1:10 dilution in duplicate. Sera, virus, and cells were diluted in the same diluent, which contained MEM (Gibco®/Invitrogen) with 0.57% bovine serum albumin Fraction V (Gibco®/Invitrogen.), 25mM HEPES buffer (Fisher Scientific), 100 mg/ml streptomycin (Fisher Scientific), and 100,000 IU penicillin (Fisher Scientific).
Using flat bottom 96-well tissue culture plates (Becton Dickinson, Franklyn Lakes, NJ), 50µl of diluted sera and 50µl of virus at 100 TCID<sub>50</sub> were mixed and incubated for 2 hours at 37°C and 5% CO<sub>2</sub>. Following incubation, 100µl of freshly trypsinized MDCK cells diluted to a concentration of 2.0x10<sup>5</sup> cells/ml was added to each well. The plates were then incubated at 37°C and 5% CO<sub>2</sub> for 24 hours. The following day, the plates were first washed twice with PBS and then the cell monolayers were fixed with cold 80% acetone for 10min. The acetone was discarded and plates left to air dry.

Following the virus neutralization procedure, an enzyme-linked immunosorbent assay (ELISA) was performed to determine if cells were infected with virus. Plates were first washed 4 times in wash buffer containing PBS with 0.5% Tween-20 (Fisher Scientific). Mouse anti-influenza A (CDC Biological Products, Atlanta, GA) primary antibody was diluted 1:4000 in PBS with 5% nonfat dry milk powder and 0.1% Tween-20. To each well, 100µl of diluted primary antibody was added and plates incubated for 1hr at room temperature. Plates were washed 4 times in wash buffer and then 100µl of secondary antibody (goat anti-mouse IgG conjugated to horseradish peroxidase, Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MD) diluted 1:2000 in a blocking buffer was added to each well. Plates incubated for another hr at room temperature and were again washed 4 times with a wash buffer. To each well, 100µl of tetramethylbenzidine peroxidase substrate 2 (TMB) (KPL) was added. After 10 minutes in the dark at room temperature, the TMB was stopped with 1N sulfuric acid (Fisher Scientific). Within 30min of stopping the reaction, absorbance was read at 450nm wavelength using an automated microplate spectrophotometer (Powerwave 340, Biotek, Winooski, VT). Average absorbance was determined for each plate individually with quadruplicate wells of virus-infected control (VC) and uninfected cell control (CC) wells. The serum antibody titer result was expressed as the reciprocal of the highest dilution of serum with absorbance value less than <i>X</i>, where <i>X</i> = [(average absorbance of VC wells) + (average absorbance of CC wells)]/2. To ensure the virus was added at the correct
TCID<sub>50</sub>, a virus back titer consisting of 8 two-fold serial dilutions run in octuplicate was only accepted when it produced positive results in the first 5-7 wells containing the lowest dilution of test virus for at least 6 of the 8 repeats. If the back titer failed, the MN assay was repeated. With a passing back titer, results for test sera were recorded. If specific antibodies were present, they inhibited the influenza virus's ability to infect the MCDK cells and the subsequent ELISA was negative for virus. Sera with at least one duplicate with a positive result at 1:10 were retested with a 2-fold serial dilution up to 1:1280 in duplicate.

**Neuraminidase inhibition (NI) assay**

Based on previous reports [119-121], a qualitative NI assay was developed to examine subjects' sera for antibodies with neuraminidase (N8) subtype specificity. The NI assay was founded on the neuraminidase's biological activity of removing N-acetyl neuraminic acid (NANA) or sialic acid from glycoproteins (Figure 2). Removing sialic acid from both the virus and infected cell after virus replication allows for the virus to release from the cell and infect new cells. For the NI assay, the source of bound NANA is fetuin, a blood protein from fetal calf serum. If the NANA is cleaved by the virus, a subsequent chain of reactions will react with the free NANA producing a chromophore: periodate oxidizes the NANA and produces β-formyl pyruvic acid; sodium arsenate neutralizes excess periodate; thiobarbituric acid reacts with β-formyl pyruvic acid at 56°C to produce a pink color. The dark pink color indicates no antibodies were present to inactivate the virus. Specific antibodies against the neuraminidase will inhibit the virus from cleaving NANA from the fetuin, and a reduced pink color is visualized.

A virus titration was first performed to determine the optimum antigen dilution, which was the highest dilution with a "medium" pink color, or the dilution prior to a reduction in pink color. Sera were heat-inactivated at 56°C for 30min then diluted 1:2.25 in PBS, per previous reports [120]. To be noted, various sera dilutions were tested and
the recommended 1:2.25 was confirmed as optimal for human sera. Positive control dog sera was diluted 1:64. Using white opaque polystyrene 96-well microtiter plates (Nunc, Rochester, NY), 25μl of test sera were added in duplicate, followed by 25μl of standardized virus. Virus control wells with PBS only as well as positive control wells employing canine sera from dogs confirmed positive with CIV infections were used as assay controls (note, due to their high reactivity, canine control sera were diluted at 1:64). Plates were covered, mixed on a microtiter plate shaker for 10-15 seconds, and incubated at room temperature for 1hr. Following incubation, 25μl of 12.5mg/ml fetuin from fetal calf serum (Sigma-Aldrich, St. Louis, MO) in 0.4M PBS (pH 5.9) was added to each well. Plates were again covered, mixed on a microtiter plate shaker for 10-15 seconds, and incubated at 37°C for 4hrs. Plates were then cooled to room temperature and 25μl of sodium m-periodate (4.28% sodium m-periodate [weight:volume], 62% concentrated phosphoric acid, and 38% water) was added to each well. Plates were covered, mixed on a microtiter plate shaker for 10-15 seconds, and incubated at room temperature for 20min. Next, 25μl of sodium m-arsenite (50% sodium m-arsenite [weight:volume] and 1.5% concentrated sulfuric acid in water) was added to each well. The addition of sodium arsenite formed a dark brown precipitate for which the plates were mixed on a microtiter plate shaker until the color faded (up to 10 minutes). Once the color faded to yellow, 100μl of 2-thiobarbituric acid (0.6% thiobarbituric acid [weight:volume] dissolved in water) was added to all wells. Plates were covered with sealing tape and then small pinholes were punctured into the tape over each well to allow for expansion. Sealed plates floated in a 56°C water bath for 1hr. Following incubation, results were analyzed for color development. A dark pink color was deemed negative, and a reduced pink or light pink color was considered positive (Figure 3). Positive results were classified as strong positive or weak positive, based on the level of color reduction.
**Immunodot blot assay:**

An immunodot blot (dot blot) was developed as a means to validate the MN and NI assay results, in the event of discrepancies. BEI Resources (Manassas, VA) manufactured a baculovirus recombinant neuraminidase (rN8) protein with an N-terminal histidine tag from an H3N8 equine influenza virus (A/equine/Pennsylvania/1/2007) (Catalog #NR-13523). Several dot blot methods were attempted.

**Coronavirus**

Respiratory swabs collected from dogs confirmed positive for canine respiratory coronavirus were kindly provided by Dr. Kathy Kurth, University of Wisconsin, Madison, WI and Dr. Edward Dubovi from Cornell University, Ithaca, NY. Human sera testing ELISA positive for various human coronaviruses was kindly provided by Dr. Samuel Dominguez at The Children’s Hospital Association, Aurora CO.

**Virus culture**

Canine respiratory coronavirus

CRCoV was cultured on the human intestinal adenocarcinoma cell line HCT-8 (ATCC catalog #CCL-224) with modified RPMI 1640 media (RPMI 1640 (Gibco®/Invitrogen), 10mM HEPES buffer (Fisher Scientific), 5% fetal bovine serum (FBS), 10% glucose, 100mM sodium pyruvate, 100 mg/ml streptomycin (Fisher Scientific), and 100,000 IU penicillin (Fisher Scientific), per previous reports [88]. Freshly trypsinized cells were washed with RPMI infection media (FBS dropped to 2%) and inoculated in suspension with 700μl of a canine swab sample diluted 1:4 in infection media for 1hr at 35°C with 5% CO2. Following rocking incubation in cell suspension, cells and virus were seeded onto one 150cm² cell culture flask (Corning, Corning, NY) and 15ml of infection media added. A mock infected flask of HCT-8 cells was included for a source of negative control cells. After 5 days at 80-90% confluency, cells were
harvested per a previous report [26]. The supernatant was first harvested and spun at 1800xg for 10min for remove cellular debris. Supernatant was aliquoted and stored. Cells were scraped into 10ml of PBS and washed twice with PBS by centrifuging at 500xg at 4°C for 10min. After the last wash, the PBS was discarded and 0.5ml of 1% Igepal (Sigma-Aldrich) per flask was added. Cells were mixed for 1hr at room temperature on a rocker. The cell lysate was then spun at 17,000xg for 30min, with the resultant supernatant collected and pelleted debris discarded.

Human coronavirus OC43

Virus was also propagated in HCT-8 cells. Cells were seeded in 150cm² cell culture flasks (Corning, Corning, NY), and upon 90% confluency, flasks were washed 3 times with plain RPMI 1640 media (Gibco®/Invitrogen); the RPMI remained on the cells for 10min during the final rinse. Residual RPMI was removed with a pipette. One milliliter of passage 8 HCoV OC43 (ATTC # VR-1558) diluted 1:4 in RPMI infection media was inoculated onto each flask. Cells were incubated with rocking for 1hr at 37°C with 5% CO₂. An additional 15ml of RPMI infection media was then added and cells were incubated at 37°C with 5% CO₂ until 70-90% CPE was visualized but a cell monolayer was still adhered (24hrs). Cells were harvested as previously described for CRCoV. A mock-infected flask was included as a negative control.

Real-time RT-PCR

Real-time reverse transcription polymerase chain reaction (qRT-PCR) performed with proprietary primers and probe provided by Dr. Kathy Kurth, University of Wisconsin, Madison WI, to detect the presence of CRCoV in the cell culture. First, RNA was extracted from the canine swab sample and culture supernatant with the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA), and from infected HCT-8 cells with the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. One step qRT-PCR was run using Superscript III Reverse Transcriptase with Platinum Taq (Invitrogen)
at the following conditions: After cDNA synthesis at 42°C for 15min followed by an initial denaturation at 95°C for 2min, 40 cycles were carried out with denaturation at 95°C for 15sec and extension at 53°C for 30sec. Cycle threshold (Ct) values were examined to determine the number of cycles required for the fluorescent signal to cross a threshold (background) level.

**Protein quantification**

Per a previous report [26], the protein concentration of the CRCoV, HCoV OC43 and negative control cell lysates was determined with the Pierce Coomassie (Bradford) colorimetric protein assay kit (Thermo Fisher Scientific/Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Optical densities of the samples were determined using the Powerwave 340 automated microplate spectrophotometer (Biotek). Referencing a standard curve with bovine serum albumin, protein concentrations (μl/ml) were estimated.

**Competitive ELISA**

A competitive ELISA using whole virus CRCoV antigen was developed by adapting previous reports of a CRCoV antigen standard ELISA for canine serum [26, 78]. The assay was first developed using HCoV OC43 virus with sera confirmed seropositive for antibodies against the virus, to determine optimal conditions. Once optimized, a standard ELISA using CRCoV antigen with the cross-reacting HCoV positive control sera was developed to ensure proper protein coating. A competitive ELISA was then performed as described below, using sera pre-incubated with HCoV-infected culture supernatant and then added to plates coated with CRCoV antigen.

Clear 96-well flat bottom, high binding Immulon® 2HB polystyrene microtiter plates (Thermo Scientific, Rochester, NY) were coated with cell lysate antigen (virus-infected cells and negative cell control, in duplicates) at a protein concentration of approximately 20μl/ml diluted in coating buffer (1L water, 1.59g sodium carbonate, and
2.93g sodium bicarbonate; pH 9.6). Plates were sealed with sealing tape and incubated overnight at 4°C for optimal protein binding. The next day, wells were washed 3 times with PBS and blocked with 300μl blocking buffer (5% nonfat dry milk powder [Carnation] in PBS) for 1hr at room temperature. Plates were washed once with PBS. Sera were first mixed 1:1 with HCoV OC43 culture supernatant and incubated at 37°C for 1hr, then 50μl of the sera mixture diluted 1:50 (final sera dilution of 1:100) in dilution buffer (5% nonfat dry milk powder and 0.05% Tween 20 (Fisher Scientific) in PBS) were added to the plates in duplicate. Plate incubated at 37°C for 1hr and were then washed 3 times in wash buffer (0.05% Tween 20 in PBS). Goat anti-human IgG peroxidase conjugate (KPL) was diluted 1:6000 in dilution buffer and 50μl were added to each well. Following a 1hr incubation at room temperature, plates were washed 3 times in wash buffer. To each well, 100μl of TMB (KPL) was added. After 10 minutes in the dark at room temperature, the TMB was stopped with 1N sulfuric acid (Fisher Scientific). Within 30min of stopping the reaction, absorbance was read at 450nm wavelength using the Powerwave 340 automated microplate spectrophotometer (Biotek). Average absorbance was determined for each sera samples individually with duplicate wells of negative control cells. Serum on CRCoV-infected cell wells with absorbance value greater than the average absorbance of control wells + 3 standard deviations were considered positive.

Immunfluorescence assay

A competitive IFA was developed to confirm positive results of the competitive ELISA, adapted from a previous report for canine serum [26]. Following the methods described above for inoculation of HCT-8 cells with CRCoV, 16-well glass chamber slide systems (Fisher Scientific) were seeded with HCT-8 cells infected in suspension with CRCoV. After 2-3 days, the cells were rinsed with PBS and fixed in methanol/acetone (2:1) for 10min at -20°C. The mixture was removed and cells then
rinsed with PBS. Sera first mixed 1:1 with HCoV OC43 culture supernatant and incubated at 37°C for 1hr, were diluted 1:50 (final sera dilution of 1:100) in salty PBS (500ml PBS with 10.227g sodium chloride) and 30μl was added to a chamber. Slides incubated at 37°C for 1hr. After 3 washes in PBS, 30μl of goat anti-human IgG FITC-conjugate diluted 1:100 in salty PBS was added to each chamber and incubated in the dark at room temperature for 1hr. After 3 washes in PBS, the chambers were removed and coverslips mounted with Vectashield with DAPI mounting medium (Vector Laboratories, Peterborough). Slides were viewed under a fluorescent microscope.

**Statistical methods**

*Brucella canis*

Results of the qualitative RSAT were dichotomized as positive or negative for binary and multivariate conditional modeling. Questionnaire data was used to identify risk factors for infection such geographical location, occupation, dog-years of exposure, exposure to whelping or sick dogs, hand-washing, and wearing protective equipment. Various canine exposures were measured, including total dog-years of exposure, specific occupations/hobbies, exposure to specific types of dogs, and exposure to whelping dogs; exposure covariates were considered as both continuous and dichotomized data. For dichotomized data, Wald chi-square or Fisher's exact tests (dependent upon how sparse was the data) were calculated to demonstrate associations between outcome and independent risk factors. Conditional logistic regression was used to examine continuous exposure data as well as multiple independent variables for their association with the dichotomous outcomes. From these procedures, odds ratio (OR) estimates and confidence intervals were ascertained for simple unadjusted comparisons (e.g., canine-exposed vs. non-exposed). Comparisons were also made after adjusting for potential confounders, and possible interaction effects between covariates were also studied. Covariates with bivariate $P$ values <0.1 were considered for inclusion in all models. To
identify the model that best predicted the outcome, final multivariable models were
designed using a saturated model and manual backwards elimination. Nested models
were compared using the likelihood ratio test. Significant differences in the maximum
likelihood estimate (p<0.05) between nested models indicated the full model provided a
better fit to the data than the reduced model. For multivariate models that were not
nested, the Akaike Information Criterion (AIC) goodness-of-fit statistic was compared,
for which the model with the lowest AIC was selected. Analysis was performed using
SAS v9.2 (SAS Institute, Cary, NC).

Influenza

Per CDC guidelines, previous infection with human influenza H3N2 virus was
defined as having an HI titer $\geq 1:40$ [10]. The proportional odds model [122] was used
for ordinal MN and NI outcomes (e.g. distribution of CIV serological titers over the range
of dilution; weak/positive NI results) to maximize the power to detect differences
between the groups. The two highest MN antibody titer levels (1:40 and 1:80) were
grouped because of sparse data; negative titers were assigned a value of 1. For the NI
assay, strong positives were assigned a category of 10, weak positives, 5, and negatives
were assigned a value of 1. For dichotomous data, an MN titer $\geq 1:10$ against the H3N8
CIV was considered as positive.

Questionnaire data was used to identify risk factors for infection such as
geographical location, canine exposure, exposure to sick dogs, hand-washing, wearing
protective equipment, etc. Canine exposure covariates were considered as both
continuous and dichotomized data. For bivariate and multivariate conditional logistic
regression, Wald chi-square or Fisher's exact tests (dependent upon how sparse the data)
were calculated to demonstrate associations between dichotomized outcomes and
independent risk factors. The proportional odds score test for proportional odds
assumption was used to examine associations between ordinal outcomes and risk factors.
From these procedures, odds ratio (OR) estimates and confidence intervals were ascertained for simple unadjusted comparisons. Comparisons were also made after adjusting for potential confounders such as age, prior influenza vaccination, and human H3 titer. Covariates with $P$ values <0.1 were considered for inclusion in all models. To identify the model that best predicted the outcome, final multivariable models were designed using a full model and manual backwards elimination. Nested models were compared using the likelihood ratio test. Significant differences in the maximum likelihood estimate ($p<0.05$) between nested models indicated the full model provided a better fit to the data than the reduced model. For multivariate models that were not nested, the Akaike Information Criterion (AIC) goodness-of-fit statistic was compared, for which the model with the lowest AIC was selected. Analysis was performed using SAS v9.2 (SAS Institute, Cary, NC).

**Coronavirus**

Student’s t-test was used to compare continuous variables, and Wald chi square test was used to compare categorical variables. Logistic regression was used to compare optical density (OD) levels between the exposure groups and ascertain odds ratios and associated confidence intervals. Analysis was performed using SAS v9.2 (SAS Institute).
Figure 1. A) Synbiotics D-Tec CB *Brucella canis* Rapid Slide Agglutination Test kit. B) RSAT procedure for detecting antibodies against *B. canis*. C) Results of the RSAT; circle on right is positive for agglutination.

Source: Synbiotics Corporation (Kansas City, Missouri)
Figure 2. The neuraminidase inhibition assay - interaction of free N-acetyl neuraminic acid (NANA) with β-formyl pyruvic acid and sodium arsenite for the formation of a pink chromophore.

Figure 3. Neuraminidase inhibition assay microtiter plate.

Note: Sera samples run in vertical duplicates. Last column includes virus control (no sera) and positive control (canine sera).
Table 2. Sample size estimates necessary to detect various odds ratios.

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Note: Assume 80% power, 5% significance level, and an exposed to non-exposed ratio of 3:1.
CHAPTER 4

RESULTS

Participants
Between 2007 and 2010, a total of 306 canine-exposed subjects and 101 non-canine exposed controls granted informed consent, completed the enrollment questionnaire and submitted a serum sample (Table 3). Demographically, the gender distribution was identical between exposure groups, but the controls tended to be younger than the exposed group (averages of 33yo and 43yo, respectively). Overall, the participants were more likely to be female (68%), and 75% resided in Iowa or Florida where the majority of enrollments took place. Canine-exposed subjects were more likely to have ever used tobacco products (OR=2.1; 95% CI, 1.2-3.7) and been exposed to horses (OR=5.6; 95% CI, 3.4-9.3); however, exposed subjects were less likely to have ever received a human influenza vaccine (OR=0.5; 95% CI, 0.3-0.9) and to have a positive human H3N2 human influenza titer (OR=0.4; 95% CI, 0.3-0.7), compared to the non-exposed group.

Exposures
Table 4 and Table 5 illustrate the work/hobbies involving close contact (approx. 3 ft) with dogs, as reported by participants (respondents were allowed to indicate more than one occupation/hobby). Those most frequently reported included veterinary staff (136), dog breeders (105), kennel staff (97), animal shelter staff (48), and dog trainers (39). Sixteen greyhound racetrack employees from 2 racetracks were included in the study population. Of the canine-exposed group, 236 (77%) reported exposure to a whelping dog at some point in their lifetime, and 105 subjects (34%) participated in dog breeding as a work or hobby. Ever having exposure to cattle, sheep, pigs, goats, or deer/elk were frequent (66%, 52%, 60%, 53%, and 36%, respectively). For all animals, the exposed
group was significantly more likely to report exposure compared to the control group (OR = 3.8; 95% CI, 2.3-6.2).

**Aim 1:** *Brucella canis*

Seroprevalence findings

Thirteen subjects tested positive for antibodies against *B. canis* by the 2ME-RSAT; 11 were among the exposed group and 2 were non-exposed controls (Table 3). Positive subjects resided in Iowa, Florida, Illinois, Minnesota, and Wisconsin (Figure 4). Examining canine exposures as continuous data (e.g. total dog-years of exposure) did not improve statistical associations; therefore, all exposure data was dichotomized. Specific occupations/hobbies were no more likely to be associated with seropositivity (Table 4). Exposure to animals susceptible to other *Brucella spp.* was not associated with a positive *B. canis* antibody result (Table 6). Upon bivariate analyses, exposure to a known positive dog had the highest odds of seropositivity among the exposure variables tested (OR=5.9; 95% CI, 1.0-25.5) (Table 7). Twenty-two subjects reported exposure to a known *B. canis* positive dog, of which 3 (14%) of them tested positive for antibodies against *B. canis*. Those exposures occurred in 1985, 2006, and 2007; 2 of the 3 were associated with kennel outbreaks. The only other covariate to have a binary association with seropositivity was residing in Iowa, for which Iowans were less likely to test positive for *B. canis* antibodies (OR=0.3; 95% CI, 0.1-1.0).

None of the seropositive subjects reported using frozen colostrum when puppies could not nurse, and no one cited experiencing any illness after contact with a sick dog. Four subjects reported accidentally getting stuck with a needle containing the bovine brucella vaccine, but none of the 4 were seropositive for *B. canis*.

Exposure to a whelping dog was only moderately associated with increased likelihood of *B. canis* seropositivity (OR=1.7; 95% CI, 0.5-5.5); however, not wearing personal protective equipment (PPE) when exposed to a whelping dog had a stronger
association with increased likelihood of seropositivity, although not significantly (OR=4.5; 95% CI, 0.8-45.3). To further examine occupational exposures, additional risk factor analyses were performed using data from the 11 seropositive exposed subjects, as the 2 control subjects did not complete the occupational portion of the questionnaire (Table 8). Breeders who did not routinely perform *B. canis* testing (either when acquiring new dogs, using dogs for breeding, selling dogs, or regular testing of the kennel) were more likely to have a positive antibody result, although not significantly (OR=2.1; 95% CI, 0.1-31.2). The number of breeding females and not wearing PPE while working with dogs were not associated with seropositivity.

The variables with a considerable association to the outcome in bivariate analyses (p <0.1) were examined with multivariate logistic regression models to control for potential confounding and interaction. When examining all study subjects, only 2 covariates were considered, and the full model did not alter the unadjusted odds (Table 9). When considering only those who reported ever being exposed to a whelping dog, both exposure to a known *B. canis* positive dog (adjusted OR=7.6; 95% CI, 1.01-48.7) and not wearing PPE when exposed to a whelping dog (adjusted OR=6.0; 95% CI, 1.02-65.0) were significantly associated with *B. canis* seropositivity when controlling for residence in Iowa (Table 10). Multiplicative interaction between final covariates in the logistic regression models were not associated with elevated antibody titers.

**Aim 2: Canine influenza virus**

**Seroprevalence findings**

A total of 76 subjects had a microneutralization assay titer ≥1:10, of which 63 (83%) were in the exposed group. For the neuraminidase inhibition assay, 75 subjects were classified as positive: 31 strong positives (87% canine-exposed) and 44 weak positives (73% canine-exposed). In all, 17 subjects tested positive for both assays, of which 14 (82%) were in the exposed group. MN and NI results did not correlate (κ =
0.05 and Pearson's $r = 0.02$) (Figure 6 and Table 11). Because the serological assays did not correlate, statistical analyses were conducted across 3 outcome levels: (1) MN titer, (2) NI result, and (3) both MN and NI results combined. Examining canine exposures as continuous data (i.e. total dog-years of exposure) did not improve statistical associations; therefore, all exposure data was dichotomized.

**Proportional odds modeling**

The proportional odds assumption was not violated when comparing MN titer results by canine exposure ($p = 0.84$) (Figure 5); therefore, analysis of ordinal MN titer results was warranted when appropriate. In addition, when examining ordinal NI results (negative < weak positive < strong positive), the proportional odds assumption was not violated for the association with canine exposure ($p = 0.19$), so when appropriate the proportional odds model was used for NI outcome.

**MN assay**

Unadjusted bivariate associations were first analyzed using conditional logistic regression (Table 12 and Table 13). Canine-exposed participants were more likely to have a positive MN titer, however, this association was not statistically significant (OR=1.7; 95% CI, 0.9-3.3). No canine-exposed occupations were more likely to be associated with a positive MN antibody titer, including greyhound racetrack employees. Older age was significantly associated with seropositivity. When analyzing participants’ ages divided into quintiles, those aged between 33-45, 46-55, and 56-78 were more likely to test positive for CIV antibodies by the MN assay when compared to those 18-25yo (OR=8.7; 95% CI, 3.1-24.0; OR=8.8; 95% CI, 3.2-24.2; and OR=3.3; 95% CI, 1.1-9.9, respectively). Two potentially confounding factors were also significantly associated with the outcome. Participants who reported ever receiving a human influenza vaccine (OR=2.1; 95% CI, 1.1-3.8) and participants who had HI assay titers $\geq 1:40$ for the H3N2
human influenza virus (OR=1.8; 95% CI, 1.1-3.1) were significantly more likely to have MN titers ≥1:10.

Considering occupational risk factors among those exposed to dogs, 2 exposures were moderately associated with CIV seropositivity, although both covariates were protective factors (Table 13). Exposed subjects who never/rarely wore personal protective equipment (PPE) while working with dogs (OR=0.6; 95% CI, 0.3-1.0), were less likely to have an elevated antibody titer against CIV compared to those who sometimes/most of the time/always wore PPE. Subjects who reported a history of examining dogs (OR=0.6; 95% CI, 0.3-1.0) were also less likely to have an elevated MN titer compared to exposed subjects who did not examine dogs.

Upon multivariate conditional logistic regression, the above associations were considered for inclusion in the final model. For the entire study population, age, canine exposure, prior influenza vaccination receipt, and a positive titer for human H3N2 influenza were considered (Table 14). The final model consisted of prior receipt of a human influenza vaccine (adjusted OR=2.5; 95% CI, 1.3-4.8), and age. Compared to study subjects aged 18-25, subjects between the ages of 33-45 and 46-55 were significantly more likely to have elevated antibody titers (adjusted OR=8.8; 95% CI, 2.8-27.4 and adjusted OR=10.3; 95% CI, 3.4-31.6, respectively).

When considering only the canine-exposed study subjects, potential risk factors associated with seropositivity were examined with a multivariate model to control for confounding (Table 15). After controlling for age and prior human influenza vaccination, examining dogs (adjusted OR=0.3; 95% CI, 0.2-0.7) and never/rarely wearing PPE when working with dogs (adjusted OR=0.4; 95% CI, 0.2-0.8) were still inversely associated with elevated CIV antibody titers.
NI assay

Unadjusted bivariate associations were first analyzed (Table 12 and Table 13). The eldest age quintile was significantly more likely to have a positive NI result, when compared to the youngest age quartile (OR=14.1; 95% CI, 5.5-35.8). Prior receipt of a human influenza vaccination was also associated with the outcome (OR=2.2; 95% CI, 1.2-4.0). Breeders were more likely to have a positive NI result (OR=1.7; 95% CI, 1.0-2.8); however, shelter staff were less likely to have a positive NI result, compared to other occupations (OR=0.4; 95% CI, 0.1-1.1). Reported exposure to poultry and horses were moderately associated with a higher odds of a positive NI result (OR=2.1; 95% CI, 1.1-3.8 and OR=1.9; 95% CI, 1.0-3.7, respectively). Residence in several states was associated with the outcome: Florida (OR=0.6; 95% CI, 0.3-1.0), Wisconsin (OR=0.4; 95% CI, 0.1-1.1), Illinois (OR=3.6; 95% CI, 1.2-10.5), and Iowa (OR=1.5; 95% CI, 0.9-2.5).

Considering occupational risk factors among those exposed to dogs, 2 exposures were significantly associated with CIV seropositivity by the NI assay (Table 13). Exposed subjects who birthed puppies (OR=2.0; 95% CI, 1.2-3.6) and subjects who did not wear gloves when caring for a sick dog (OR=2.5; 95% CI, 1.3-4.8) had higher odds of seropositivity.

Upon multivariate conditional logistic regression for the entire study population, the above mentioned associations were considered for inclusion in the final model, including age, working at a shelter, breeding dogs, prior influenza vaccination, exposure to poultry and horses, and residing in Illinois (Table 16). When including influenza vaccine receipt, older age (adjusted OR=16.0; 95% CI, 5.7-45.2) and residing in Illinois (adjusted OR=4.7; 95% CI, 1.3-16.6) were significantly associated with a positive NI result. When considering occupational risk factors for only subjects who reported exposure to a sick dog, after adjusting for age and residing in Illinois, birthing puppies and not wearing gloves lost significance and were not considered further (Table 17).
**MN & NI assays**

Lastly, unadjusted bivariate associations were analyzed for subjects who tested seropositive by both the MN assay (titer ≥1:10) and NI assay (weak/strong positive) (Table 12 and Table 13). For all study subjects, only the age group 46-55 (OR=8.7; 95% CI, 1.1-396) and poultry exposure (OR=0.3; 95% CI 0.1-1.0) were associated with the outcome. For only canine-exposed subjects, no occupational risk factors analyzed were associated by binary comparisons with the outcome (Table 13). When employing multivariate logistic regression, both age (46-55yo) and poultry exposure remained significantly associated with dual MN and NI seropositive results (adjusted OR=12.2; 95% CI 1.4-585 and adjusted OR=0.2; 95% CI, 0.1-0.8, respectively) (Table 18). Subjects aged 46-55 were more likely to have dual seropositive results, while subjects who reported exposure to poultry were less likely than those not exposed to poultry to have dual CIV seropositivity.

**Aim 3: Canine respiratory coronavirus**

**Competitive CRCoV ELISA**

Along with HCoV OC43, CRCoV was successfully cultured in HCT-8 cells (Figure 7). Relying on cross-reacting antibodies, the ELISA was first developed with HCoV OC43 for canine sera using sera collected from a dog PCR+ for CRCoV, because culturing CRCoV was difficult and unpredictable. Once successful, the assay was then adapted to human sera using sera positive for antibodies against HCoVs OC43, HKU1, NL63, and 229E. Next, the assay was replicated with CRCoV first with the canine serum and then the human sera. Upon proof that enough CRCoV antigen was present to react with the canine antibodies and cross-reacting human antibodies, a competitive ELISA was developed to eliminate HCoV cross-reacting antibodies. The competitive CRCoV ELISA was deemed successful when a known OC43-positive human sera was ELISA positive when un-competed and then ELISA negative after competition. Canine sera was
also blocked with HCoV, and it remained positive with the CRCoV ELISA; however, the titer was lower than when un-competed.

Seroprevalence findings

Based upon results of the competitive ELISA, there was no serological evidence of previous exposure to CRCoV among the study population. The frequency of mean OD levels indicated a normal distribution with no apparent outliers (Figure 8 and Figure 9). In addition, there was no significant difference in the mean OD levels ([average of the test wells] - [average of the negative control wells + 3 standard deviations]) between the 2 groups (Figure 3). The mean OD was -0.03 for both canine-exposed subjects and non-exposed controls, with no significant difference between the groups, examining both continuous OD data and OD levels categorized into quartiles (Table 19).

Competitive CRCoV IFA

Analogous to the ELISA development, a competitive CRCoV IFA was similarly developed to confirm positive ELISA results; however, because there were no compelling results from the ELISA, this assay was not employed.
Figure 4. Geographical distribution by home residence for study subjects with elevated antibodies against *Brucella canis*.

Note: Dark-shaded states denotes seropositive subjects identified and includes number of seropositive and corresponding percent of the subjects enrolled from the state.
Figure 5. Schematic illustration of the proportional odds assumptions using microneutralization assay titer results for the 306 canine-exposed and 101 non-canine exposed study participants.

Figure 6. Scatter plot matrix of microneutralization assay titer results\textsuperscript{a} and neuraminidase inhibition assay\textsuperscript{b} results.

Note: Graphic created with SAS v9.2 (SAS Institute, Cary, NC)

\textsuperscript{a} MN results provided as reciprocal of antibody titer.

\textsuperscript{b} NI results: 1 = weak positive; 2 = strong positive.
Figure 7. A) HCT-8 cells infected with canine respiratory coronavirus. B) Uninfected HCT-8 cells.
Figure 8. Frequency distribution of mean optical density (OD) results for the competitive canine respiratory coronavirus ELISA.

Note: (n=401).
Figure 9. Plot of mean optical density (OD) results for the competitive canine respiratory coronavirus ELISA between canine exposed and unexposed study groups.
Table 3. Unadjusted odds ratios for demographics and serological results for canine exposed against unexposed enrollees using binary conditional logistic regression.

<table>
<thead>
<tr>
<th>Covariate</th>
<th>N (n=407)</th>
<th>Exposed (n=301)</th>
<th>Control (n=106)</th>
<th>Unadjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56-78</td>
<td>78</td>
<td>69 (22.6)</td>
<td>9 (9.0)</td>
<td>7.7 (3.4-17.3)</td>
</tr>
<tr>
<td>46-55</td>
<td>85</td>
<td>76 (24.9)</td>
<td>9 (9.0)</td>
<td>8.4 (3.8-19.0)</td>
</tr>
<tr>
<td>33-45</td>
<td>77</td>
<td>62 (20.3)</td>
<td>15 (15.0)</td>
<td>4.1 (2.0-8.4)</td>
</tr>
<tr>
<td>26-32</td>
<td>79</td>
<td>55 (18.0)</td>
<td>24 (24.0)</td>
<td>2.3 (1.2-4.3)</td>
</tr>
<tr>
<td>18-25</td>
<td>86</td>
<td>43 (14.1)</td>
<td>43 (43.0)</td>
<td>Ref</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>129</td>
<td>97 (31.7)</td>
<td>32 (31.7)</td>
<td>1.0 (0.6-1.6)</td>
</tr>
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<td>Female</td>
<td>278</td>
<td>209 (68.3)</td>
<td>69 (68.3)</td>
<td>Ref</td>
</tr>
<tr>
<td>Ever used tobacco products&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>119</td>
<td>99 (34.5)</td>
<td>20 (19.8)</td>
<td>2.1 (1.2-3.7)</td>
</tr>
<tr>
<td>No</td>
<td>269</td>
<td>188 (65.5)</td>
<td>81 (80.2)</td>
<td>Ref</td>
</tr>
<tr>
<td>Exposed to domestic animals&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>301</td>
<td>242 (80.4)</td>
<td>50 (52.1)</td>
<td>3.8 (2.3-6.2)</td>
</tr>
<tr>
<td>No</td>
<td>96</td>
<td>59 (19.6)</td>
<td>46 (47.9)</td>
<td>Ref</td>
</tr>
<tr>
<td>Exposed to horses&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Yes</td>
<td>288</td>
<td>246 (82.3)</td>
<td>42 (45.2)</td>
<td>5.6 (3.4-9.3)</td>
</tr>
<tr>
<td>No</td>
<td>104</td>
<td>53 (17.7)</td>
<td>51 (54.8)</td>
<td>Ref</td>
</tr>
<tr>
<td>Ever received human influenza vaccine&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Yes</td>
<td>230</td>
<td>161 (57.7)</td>
<td>69 (72.6)</td>
<td>0.5 (0.3-0.9)</td>
</tr>
<tr>
<td>No</td>
<td>144</td>
<td>118 (42.3)</td>
<td>26 (27.4)</td>
<td>Ref</td>
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<tr>
<td>B. canis serology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>13</td>
<td>11 (3.6)</td>
<td>2 (2.0)</td>
<td>1.8 (0.4-17.4)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Negative</td>
<td>394</td>
<td>295 (96.4)</td>
<td>99 (98.0)</td>
<td>Ref</td>
</tr>
<tr>
<td>Human H3N2 influenza titer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥1:40</td>
<td>106</td>
<td>66 (21.9)</td>
<td>40 (40.0)</td>
<td>0.4 (0.3-0.7)</td>
</tr>
<tr>
<td>&lt;1:40</td>
<td>296</td>
<td>236 (78.2)</td>
<td>60 (60.0)</td>
<td>Ref</td>
</tr>
<tr>
<td>CIV meroneutralization assay titer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥1:10</td>
<td>76</td>
<td>63 (20.6)</td>
<td>13 (12.9)</td>
<td>1.8 (0.9-3.3)</td>
</tr>
<tr>
<td>&lt;1:10</td>
<td>331</td>
<td>243 (79.4)</td>
<td>88 (87.1)</td>
<td>Ref</td>
</tr>
<tr>
<td>CIV neuraminidase inhibition assay&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>75</td>
<td>59 (19.4)</td>
<td>16 (15.8)</td>
<td>1.3 (0.7-2.3)</td>
</tr>
<tr>
<td>Negative</td>
<td>330</td>
<td>245 (80.6)</td>
<td>85 (84.2)</td>
<td>Ref</td>
</tr>
<tr>
<td>CIV serological assay results combined</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MN and NI assays positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥1</td>
<td>17</td>
<td>14 (4.6)</td>
<td>3 (3.0)</td>
<td>1.6 (0.4-8.7)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>At least 1 negative</td>
<td>390</td>
<td>292 (95.4)</td>
<td>98 (97.0)</td>
<td>Ref</td>
</tr>
</tbody>
</table>

<sup>a</sup>Covariate has some missing data.

<sup>b</sup>Exact conditional logistic regression method used.
Table 4. Occupational exposures as cited by subjects and corresponding *B. canis* 2ME-RSAT serology using exact conditional logistic regression.

<table>
<thead>
<tr>
<th>Occupation</th>
<th>N</th>
<th>RSAT+ (%)</th>
<th>Unadjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veterinary staff</td>
<td>136</td>
<td>4 (2.9)</td>
<td>0.9 (0.2-3.2)</td>
</tr>
<tr>
<td>Breeder</td>
<td>105</td>
<td>5 (4.8)</td>
<td>1.8 (0.5-6.5)</td>
</tr>
<tr>
<td>Kennel staff</td>
<td>97</td>
<td>4 (4.1)</td>
<td>1.4 (0.3-5.3)</td>
</tr>
<tr>
<td>Shelter staff</td>
<td>48</td>
<td>1 (2.1)</td>
<td>0.6 (0-4.3)</td>
</tr>
<tr>
<td>Trainer</td>
<td>39</td>
<td>2 (5.1)</td>
<td>1.8 (0.2-8.5)</td>
</tr>
<tr>
<td>Groomer</td>
<td>23</td>
<td>1 (4.3)</td>
<td>1.4 (0-10.4)</td>
</tr>
<tr>
<td>Racetrack staff</td>
<td>16</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>Dog show handler</td>
<td>13</td>
<td>1 (7.7)</td>
<td>2.6 (0-20.9)</td>
</tr>
<tr>
<td>Owner/Hobbyist</td>
<td>7</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>Researcher</td>
<td>2</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>Pet store staff</td>
<td>1</td>
<td>0</td>
<td>--</td>
</tr>
</tbody>
</table>

*Subjects allowed to cite multiple occupations.*
Table 5. Occupational and hobby exposures as cited by subjects and corresponding canine influenza virus serology using conditional logistic regression.

<table>
<thead>
<tr>
<th>Occupationa</th>
<th>N</th>
<th>MN titer ≥1:10 (%)</th>
<th>Unadjusted OR (95% CI)</th>
<th>+NI (%)b</th>
<th>Unadjusted OR (95% CI)</th>
<th>+MN &amp; +NI (%)</th>
<th>Unadjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veterinary staff</td>
<td>136</td>
<td>26 (19.1)</td>
<td>1.0 (0.6-1.7)c</td>
<td>26 (19.1)</td>
<td>1.1 (0.6-1.8)c</td>
<td>7 (5.2)</td>
<td>1.4 (0.5-3.8)d</td>
</tr>
<tr>
<td>Breeder</td>
<td>105</td>
<td>24 (22.9)</td>
<td>1.4 (0.8-2.4)c</td>
<td>25 (24.3)</td>
<td>1.7 (1.0-2.8)c</td>
<td>5 (4.8)</td>
<td>1.2 (0.3-3.8)e</td>
</tr>
<tr>
<td>Kennel staff</td>
<td>97</td>
<td>14 (14.4)</td>
<td>0.7 (0.4-1.3)c</td>
<td>17 (17.5)</td>
<td>0.9 (0.5-1.6)c</td>
<td>6 (6.2)</td>
<td>1.8 (0.5-5.5)e</td>
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<tr>
<td>Shelter staff</td>
<td>48</td>
<td>5 (10.4)</td>
<td>0.5 (0.2-1.2)d</td>
<td>4 (8.5)</td>
<td>0.4 (0.1-1.1)d</td>
<td>2 (4.2)</td>
<td>1.0 (0.1-4.5)e</td>
</tr>
<tr>
<td>Trainer</td>
<td>39</td>
<td>8 (20.5)</td>
<td>1.1 (0.5-2.6)d</td>
<td>9 (23.1)</td>
<td>1.3 (0.6-2.9)c</td>
<td>1 (2.6)</td>
<td>0.6 (0.0-4.0)e</td>
</tr>
<tr>
<td>Groomer</td>
<td>23</td>
<td>4 (17.4)</td>
<td>0.9 (0.2-2.9)e</td>
<td>3 (13.0)</td>
<td>0.6 (0.1-2.3)e</td>
<td>1 (4.4)</td>
<td>1.0 (0.0-7.4)e</td>
</tr>
<tr>
<td>Racetrack staff</td>
<td>16</td>
<td>3 (18.8)</td>
<td>1.0 (0.2-3.8)e</td>
<td>1 (6.3)</td>
<td>0.3 (0.1-1.9)e</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Dog show handler</td>
<td>13</td>
<td>3 (23.1)</td>
<td>1.3 (0.2-5.3)e</td>
<td>3 (23.1)</td>
<td>1.3 (0.2-5.4)e</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Owner/Hobbyist</td>
<td>7</td>
<td>3 (42.9)</td>
<td>3.3 (0.5-20.2)e</td>
<td>2 (28.6)</td>
<td>1.8 (0.2-11.1)e</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Researcher</td>
<td>2</td>
<td>1 (50.0)</td>
<td>4.4 (0.1-346)e</td>
<td>1 (50.0)</td>
<td>4.4 (0.1-350)e</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Pet store staff</td>
<td>1</td>
<td>1 (100.0)</td>
<td>4.4 (0.1-∞)e</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Note: Exposure variables dichotomized as exposed/unexposed; MN = Microneutralization assay; NI = Neuraminidase inhibition assay

a Subjects allowed to cite multiple occupations.

b Results missing for 2 subjects.

c Proportional odds model used.

d Wald chi-square test used.

e Fisher's exact test used.
Table 6. Unadjusted odds ratio for an association between animal exposures and *B. canis* 2ME-RSAT result using exact conditional logistic regression method.

<table>
<thead>
<tr>
<th>Animal Exposure&lt;sup&gt;a&lt;/sup&gt;</th>
<th>N (n=407)</th>
<th>RSAT+ (%)</th>
<th>Unadjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cattle</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>257</td>
<td>8 (61.5)</td>
<td>0.8 (0.2-3.2)</td>
</tr>
<tr>
<td>No</td>
<td>132</td>
<td>5 (38.5)</td>
<td>Ref</td>
</tr>
<tr>
<td><strong>Sheep</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>199</td>
<td>7 (58.3)</td>
<td>1.3 (0.3-5.2)</td>
</tr>
<tr>
<td>No</td>
<td>181</td>
<td>5 (41.2)</td>
<td>Ref</td>
</tr>
<tr>
<td><strong>Pigs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>229</td>
<td>6 (50.0)</td>
<td>0.7 (0.2-2.1)</td>
</tr>
<tr>
<td>No</td>
<td>151</td>
<td>6 (50.0)</td>
<td>Ref</td>
</tr>
<tr>
<td><strong>Goats</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>202</td>
<td>4 (33.3)</td>
<td>0.4 (0.1-1.7)</td>
</tr>
<tr>
<td>No</td>
<td>180</td>
<td>8 (66.7)</td>
<td>Ref</td>
</tr>
<tr>
<td><strong>Deer/Elk</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>133</td>
<td>4 (36.4)</td>
<td>1.0 (0.3-3.5)</td>
</tr>
<tr>
<td>No</td>
<td>234</td>
<td>7 (63.6)</td>
<td>Ref</td>
</tr>
</tbody>
</table>

<sup>a</sup> Covariates have some missing data.
Table 7. Unadjusted odds ratio for risk factors associated with a positive *B. canis* 2ME-RSAT result using exact conditional logistic regression.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>N (n=407)</th>
<th>RSAT+ (%)</th>
<th>Unadjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>129</td>
<td>6 (46.2)</td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>278</td>
<td>7 (53.9)</td>
</tr>
<tr>
<td>Age group (yrs)(^a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age group (yrs)(^a)</td>
<td>49-78</td>
<td>137</td>
<td>3 (23.1)</td>
</tr>
<tr>
<td>Age group (yrs)(^a)</td>
<td>30-48</td>
<td>138</td>
<td>7 (53.9)</td>
</tr>
<tr>
<td>Age group (yrs)(^a)</td>
<td>18-29</td>
<td>130</td>
<td>3 (23.1)</td>
</tr>
<tr>
<td>Exposed to dogs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exposed to dogs</td>
<td>Yes</td>
<td>306</td>
<td>11 (84.6)</td>
</tr>
<tr>
<td>Exposed to dogs</td>
<td>No</td>
<td>101</td>
<td>2 (15.4)</td>
</tr>
<tr>
<td>Dog breeder</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog breeder</td>
<td>Yes</td>
<td>105</td>
<td>5 (38.5)</td>
</tr>
<tr>
<td>Dog breeder</td>
<td>No</td>
<td>302</td>
<td>8 (61.5)</td>
</tr>
<tr>
<td>Only dog breeder(^a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Only dog breeder(^a)</td>
<td>Yes</td>
<td>37</td>
<td>3 (23.1)</td>
</tr>
<tr>
<td>Only dog breeder(^a)</td>
<td>No</td>
<td>369</td>
<td>10 (76.9)</td>
</tr>
<tr>
<td>Exposed to whelping dog</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exposed to whelping dog</td>
<td>Yes</td>
<td>241</td>
<td>9 (69.2)</td>
</tr>
<tr>
<td>Exposed to whelping dog</td>
<td>No</td>
<td>166</td>
<td>4 (30.8)</td>
</tr>
<tr>
<td>Exposed to known <em>B. canis</em> positive dog</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exposed to known <em>B. canis</em> positive dog</td>
<td>Yes</td>
<td>22</td>
<td>3 (23.1)</td>
</tr>
<tr>
<td>Exposed to known <em>B. canis</em> positive dog</td>
<td>No/Unknown</td>
<td>385</td>
<td>10 (76.9)</td>
</tr>
<tr>
<td>Resident of Florida</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resident of Florida</td>
<td>Yes</td>
<td>115</td>
<td>5 (38.5)</td>
</tr>
<tr>
<td>Resident of Florida</td>
<td>No</td>
<td>292</td>
<td>8 (61.5)</td>
</tr>
<tr>
<td>Resident of Wisconsin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resident of Wisconsin</td>
<td>Yes</td>
<td>45</td>
<td>2 (15.4)</td>
</tr>
<tr>
<td>Resident of Minnesota</td>
<td>No</td>
<td>362</td>
<td>11 (84.6)</td>
</tr>
<tr>
<td>Resident of Minnesota</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resident of Minnesota</td>
<td>Yes</td>
<td>6</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>Resident of Minnesota</td>
<td>No</td>
<td>401</td>
<td>12 (92.3)</td>
</tr>
<tr>
<td>Resident of Illinois</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resident of Illinois</td>
<td>Yes</td>
<td>13</td>
<td>2 (15.4)</td>
</tr>
<tr>
<td>Resident of Iowa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resident of Iowa</td>
<td>Yes</td>
<td>206</td>
<td>3 (23.1)</td>
</tr>
<tr>
<td>Resident of Iowa</td>
<td>No</td>
<td>201</td>
<td>10 (76.9)</td>
</tr>
</tbody>
</table>

\(^a\) Covariate has some missing data.  \(^b\) Wald chi-square test used.
Table 8. Unadjusted odds ratio for risk factors associated with a positive *B. canis* 2ME-RSAT among canine exposed study subjects using exact conditional logistic regression.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>N (n=407)</th>
<th>RSAT+ (%)</th>
<th>Unadjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of breeding females cited by current breeders</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥2</td>
<td>67</td>
<td>4 (80.0)</td>
<td>1.3 (0.1-68.7)</td>
</tr>
<tr>
<td>0-1</td>
<td>22</td>
<td>1 (20.0)</td>
<td>Ref</td>
</tr>
<tr>
<td>Dogs tested for <em>B. canis</em> by breeder&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>26</td>
<td>2 (50.0)</td>
<td>2.1 (0.1-31.2)</td>
</tr>
<tr>
<td>Yes</td>
<td>54</td>
<td>2 (50.0)</td>
<td>Ref</td>
</tr>
<tr>
<td>Frequency of wearing PPE while working with dogs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never/Rarely</td>
<td>171</td>
<td>4 (40.0)</td>
<td>0.4 (0.1-1.9)</td>
</tr>
<tr>
<td>Sometimes/Most of the time/Always</td>
<td>118</td>
<td>6 (60.0)</td>
<td>Ref</td>
</tr>
<tr>
<td>Wore PPE when exposed to whelping dog</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>103</td>
<td>7 (77.8)</td>
<td>4.5 (0.8-45.3)</td>
</tr>
<tr>
<td>Yes</td>
<td>126</td>
<td>2 (22.2)</td>
<td>Ref</td>
</tr>
</tbody>
</table>

Note: PPE = Personal protective equipment

<sup>a</sup> Covariate has some missing data.
Table 9. Risk factors associated with a positive *B. canis* 2ME-RSAT result using multivariate exact conditional logistic regression for all study subjects.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Unadjusted OR (95% CI)</th>
<th>Final Model OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposed to known <em>B. canis</em> positive dog(^a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5.9 (1.0-25.5)</td>
<td>5.4 (0.9-23.8)</td>
</tr>
<tr>
<td>No/Unknown</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Resident of Iowa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.3 (0.1-1.0)(^b)</td>
<td>0.3 (0.1-1.2)</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>Ref</td>
</tr>
</tbody>
</table>

\(^a\) Covariate has some missing data. \(^b\) Wald chi-square test used.
Table 10. Risk factors associated with a positive *B. canis* 2ME-RSAT result using multivariate exact conditional logistic regression for only those exposed to a whelping dog.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Unadjusted OR (95% CI)</th>
<th>Final Model OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposed to known <em>B. canis</em> positive dog</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5.9 (0.9-30.7)</td>
<td>7.6 (1.01-48.7)</td>
</tr>
<tr>
<td>No/Unknown</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Resident of Iowa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.3 (0-1.5)</td>
<td>3.0 (0.5-31.3)</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Wear PPE when exposed to whelping dog</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>4.5 (0.8-45.3)</td>
<td>6.0 (1.02-65.0)</td>
</tr>
<tr>
<td>Yes</td>
<td>Ref</td>
<td>Ref</td>
</tr>
</tbody>
</table>
Table 11. Frequency comparison of microneutralization assay titer results and neuraminidase inhibition assay results for the study population.

<table>
<thead>
<tr>
<th>Neuraminidase inhibition assay</th>
<th>0</th>
<th>1:10</th>
<th>1:20</th>
<th>1:40</th>
<th>1:80</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>271</td>
<td>29</td>
<td>22</td>
<td>6</td>
<td>2</td>
<td>330</td>
</tr>
<tr>
<td>Weak positive</td>
<td>33</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>44</td>
</tr>
<tr>
<td>Strong Positive</td>
<td>25</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>329</td>
<td>39</td>
<td>26</td>
<td>8</td>
<td>3</td>
<td>405</td>
</tr>
</tbody>
</table>
Table 12. Unadjusted odds ratio for canine influenza virus seropositivity and associated risk factors using conditional logistic regression.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>N (n=407)</th>
<th>MN titer ≥1:10 (%)</th>
<th>Unadjusted OR (95% CI)</th>
<th>+NI (%)</th>
<th>Unadjusted OR (95% CI)</th>
<th>+MN and +NI (%)</th>
<th>Unadjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>129</td>
<td>27 (35.5)</td>
<td>1.3 (0.7-2.1)</td>
<td>27 (36.0)</td>
<td>1.2 (0.7-2.1)</td>
<td>2 (11.8)</td>
<td>0.3 (0.1-1.2)</td>
</tr>
<tr>
<td>Female</td>
<td>278</td>
<td>49 (64.5)</td>
<td>Ref</td>
<td>48 (64.0)</td>
<td>Ref</td>
<td>15 (88.2)</td>
<td>Ref</td>
</tr>
<tr>
<td>Age group (yrs)^c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56-78</td>
<td>78</td>
<td>13 (17.1)</td>
<td>3.3 (1.1-9.9)^a</td>
<td>39 (52.7)</td>
<td>14.1 (5.5-35.8)^a</td>
<td>3 (17.7)</td>
<td>3.4 (0.3-181)^d</td>
</tr>
<tr>
<td>46-55</td>
<td>85</td>
<td>30 (39.5)</td>
<td>8.8 (3.2-24.2)^a</td>
<td>13 (17.6)</td>
<td>2.4 (0.9-6.7)^a</td>
<td>8 (47.1)</td>
<td>8.7 (1.1-396)^a</td>
</tr>
<tr>
<td>33-45</td>
<td>77</td>
<td>26 (34.2)</td>
<td>8.7 (3.1-24.0)^a</td>
<td>6 (8.1)</td>
<td>1.1 (0.3-3.6)^a</td>
<td>3 (17.7)</td>
<td>3.4 (0.3-183)^d</td>
</tr>
<tr>
<td>26-32</td>
<td>79</td>
<td>2 (2.6)</td>
<td>0.4 (0.1-2.3)^a</td>
<td>10 (13.5)</td>
<td>2.0 (0.7-5.6)^a</td>
<td>2 (11.8)</td>
<td>2.2 (0.1-131)^d</td>
</tr>
<tr>
<td>18-25</td>
<td>86</td>
<td>5 (6.6)</td>
<td>Ref</td>
<td>6 (8.1)</td>
<td>Ref</td>
<td>1 (5.9)</td>
<td>Ref</td>
</tr>
<tr>
<td>Canine exposure</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>306</td>
<td>63 (82.9)</td>
<td>1.7 (0.9-3.3)^a</td>
<td>59 (78.7)</td>
<td>1.3 (0.7-2.4)^a</td>
<td>14 (82.4)</td>
<td>1.6 (0.4-8.7)^d</td>
</tr>
<tr>
<td>No</td>
<td>101</td>
<td>13 (17.1)</td>
<td>Ref</td>
<td>16 (21.3)</td>
<td>Ref</td>
<td>3 (177)</td>
<td>Ref</td>
</tr>
<tr>
<td>Ever used tobacco products^c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>119</td>
<td>20 (28.6)</td>
<td>0.9 (0.5-1.5)^a</td>
<td>24 (32.0)</td>
<td>1.2 (0.7-2.1)^a</td>
<td>4 (25.0)</td>
<td>0.7 (0.2-2.5)^d</td>
</tr>
<tr>
<td>No</td>
<td>269</td>
<td>50 (71.4)</td>
<td>Ref</td>
<td>51 (68.8)</td>
<td>Ref</td>
<td>12 (75.0)</td>
<td>Ref</td>
</tr>
<tr>
<td>Ever received a human influenza vaccine^c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>230</td>
<td>50 (74.6)</td>
<td>2.1 (1.1-3.8)^b</td>
<td>52 (75.4)</td>
<td>2.2 (1.2-4.0)^a</td>
<td>10 (66.7)</td>
<td>1.3 (0.4-3.8)^b</td>
</tr>
<tr>
<td>No</td>
<td>144</td>
<td>17 (25.4)</td>
<td>Ref</td>
<td>17 (24.6)</td>
<td>Ref</td>
<td>5 (33.3)</td>
<td>Ref</td>
</tr>
<tr>
<td>Human H3N2 influenza titer^c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥1:40</td>
<td>106</td>
<td>27 (36.5)</td>
<td>1.8 (1.1-3.1)^b</td>
<td>22 (29.7)</td>
<td>1.2 (0.7-2.0)^a</td>
<td>5 (29.4)</td>
<td>1.2 (0.4-3.4)^d</td>
</tr>
<tr>
<td>&lt;1:40</td>
<td>296</td>
<td>47 (63.5)</td>
<td>Ref</td>
<td>52 (70.3)</td>
<td>Ref</td>
<td>12 (70.6)</td>
<td>Ref</td>
</tr>
<tr>
<td>Exposed to known CIV+ dog^c</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>23</td>
<td>5 (6.6)</td>
<td>1.2 (0.3-3.6)^d</td>
<td>5 (6.7)</td>
<td>1.2 (0.3-3.6)^d</td>
<td>1 (5.9)</td>
<td>1.0 (0.1-41.9)^d</td>
</tr>
<tr>
<td>No</td>
<td>384</td>
<td>71 (93.4)</td>
<td>Ref</td>
<td>70 (93.3)</td>
<td>Ref</td>
<td>16 (94.1)</td>
<td>Ref</td>
</tr>
<tr>
<td>Ever exposed to a sick dog^c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>288</td>
<td>59 (77.6)</td>
<td>1.5 (0.8-2.6)^a</td>
<td>57 (76.0)</td>
<td>1.4 (0.8-2.4)^a</td>
<td>13 (76.5)</td>
<td>1.3 (0.4-5.6)^d</td>
</tr>
<tr>
<td>No</td>
<td>115</td>
<td>17 (22.4)</td>
<td>Ref</td>
<td>18 (24.0)</td>
<td>Ref</td>
<td>4 (23.5)</td>
<td>Ref</td>
</tr>
<tr>
<td>Exposed to poultry^c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>245</td>
<td>44 (62.9)</td>
<td>0.9 (0.5-1.5)^d</td>
<td>54 (72.6)</td>
<td>2.1 (1.1-3.8)^a</td>
<td>6 (40.0)</td>
<td>0.3 (0.1-1.0)^b</td>
</tr>
<tr>
<td>No</td>
<td>128</td>
<td>26 (37.1)</td>
<td>Ref</td>
<td>16 (27.4)</td>
<td>Ref</td>
<td>9 (60.0)</td>
<td>Ref</td>
</tr>
<tr>
<td>Category</td>
<td>Exposure</td>
<td>MN (%)</td>
<td>OR (95% CI)</td>
<td>P-value</td>
<td>Ref (%)</td>
<td>OR (95% CI)</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>----------</td>
<td>--------</td>
<td>-------------</td>
<td>---------</td>
<td>---------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>Exposed to horses</td>
<td>Yes</td>
<td>288</td>
<td>55 (74.3)</td>
<td>1.0 (0.6-1.8)$^a$</td>
<td>60 (81.1)</td>
<td>1.9 (1.0-3.7)$^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>104</td>
<td>19 (25.7)</td>
<td>Ref</td>
<td>14 (18.9)</td>
<td>Ref</td>
<td></td>
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<tr>
<td>Racetrack employee</td>
<td>Yes</td>
<td>16</td>
<td>3 (4.0)</td>
<td>1.0 (0.2-3.8)$^d$</td>
<td>1 (1.3)</td>
<td>0.3 (0.1-1.9)$^d$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>391</td>
<td>73 (96.0)</td>
<td>Ref</td>
<td>74 (98.7)</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>Resident of Florida</td>
<td>Yes</td>
<td>115</td>
<td>17 (22.4)</td>
<td>0.7 (0.4-1.3)$^a$</td>
<td>15 (20.0)</td>
<td>0.6 (0.3-1.0)$^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>292</td>
<td>59 (77.6)</td>
<td>Ref</td>
<td>60 (80.0)</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>Resident of Wisconsin</td>
<td>Yes</td>
<td>45</td>
<td>8 (10.5)</td>
<td>0.9 (0.4-2.0)$^a$</td>
<td>5 (6.7)</td>
<td>0.4 (0.1-1.1)$^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>362</td>
<td>68 (89.5)</td>
<td>Ref</td>
<td>70 (93.3)</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>Resident of Illinois</td>
<td>Yes</td>
<td>13</td>
<td>1 (1.3)</td>
<td>0.4 (0.2-2.5)$^d$</td>
<td>5 (6.7)</td>
<td>3.6 (1.2-10.5)$^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>394</td>
<td>75 (98.7)</td>
<td>Ref</td>
<td>70 (93.3)</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>Resident of Iowa</td>
<td>Yes</td>
<td>206</td>
<td>42 (55.3)</td>
<td>1.3 (0.8-2.1)$^a$</td>
<td>44 (58.7)</td>
<td>1.5 (0.9-2.5)$^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>201</td>
<td>34 (44.7)</td>
<td>Ref</td>
<td>31 (41.3)</td>
<td>Ref</td>
<td></td>
</tr>
</tbody>
</table>

Note: MN=Microneutralization assay; NI=Neuraminidase inhibition assay.

$^a$ Proportional odds model used.

$^b$ Wald chi-square test used.

$^c$ Covariate has some missing data.

$^d$ Fisher’s exact test used.
Table 13. Unadjusted odds ratio for canine influenza virus seropositivity and associated risk factors using conditional logistic regression for canine-exposed subjects only.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>N (n=407)</th>
<th>MN titer ≥1:10 (%)</th>
<th>Unadjusted OR (95% CI)</th>
<th>+NI (%)</th>
<th>Unadjusted OR (95% CI)</th>
<th>+MN and +NI (%)</th>
<th>Unadjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wore PPE when working with dogs&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never/Rarely</td>
<td>171</td>
<td>27 (47.4)</td>
<td>0.6 (0.3-1.0)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34 (61.8)</td>
<td>1.2 (0.7-2.2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5 (41.7)</td>
<td>0.5 (0.1-1.8)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sometimes/Most of the time/Always</td>
<td>118</td>
<td>30 (52.6)</td>
<td>Ref</td>
<td>21 (38.2)</td>
<td>Ref</td>
<td>7 (58.3)</td>
<td>Ref</td>
</tr>
<tr>
<td>Examined dogs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>169</td>
<td>28 (44.4)</td>
<td>0.6 (0.3-1.0)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34 (57.6)</td>
<td>1.1 (0.6-2.0)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6 (42.9)</td>
<td>0.6 (0.2-1.8)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>137</td>
<td>35 (55.6)</td>
<td>Ref</td>
<td>25 (42.4)</td>
<td>Ref</td>
<td>8 (57.1)</td>
<td>Ref</td>
</tr>
<tr>
<td>Treated ill dogs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>166</td>
<td>30 (47.6)</td>
<td>0.7 (0.4-1.2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32 (54.2)</td>
<td>1.0 (0.6-1.7)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5 (35.7)</td>
<td>0.5 (0.1-1.4)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>140</td>
<td>33 (52.4)</td>
<td>Ref</td>
<td>27 (45.8)</td>
<td>Ref</td>
<td>9 (64.3)</td>
<td>Ref</td>
</tr>
<tr>
<td>Vaccinated dogs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>155</td>
<td>28 (44.4)</td>
<td>0.7 (0.4-1.2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32 (54.2)</td>
<td>1.1 (0.6-2.0)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6 (42.9)</td>
<td>0.7 (0.2-2.1)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>151</td>
<td>35 (55.6)</td>
<td>Ref</td>
<td>27 (45.8)</td>
<td>Ref</td>
<td>8 (57.1)</td>
<td>Ref</td>
</tr>
<tr>
<td>Disinfected equipment/areas exposed to dogs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>229</td>
<td>43 (68.3)</td>
<td>0.7 (0.4-1.3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40 (67.8)</td>
<td>0.6 (0.3-1.1)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9 (64.3)</td>
<td>0.6 (0.2-2.3)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>77</td>
<td>20 (31.8)</td>
<td>Ref</td>
<td>19 (32.2)</td>
<td>Ref</td>
<td>5 (35.7)</td>
<td>Ref</td>
</tr>
<tr>
<td>Birthed puppies</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>124</td>
<td>26 (41.3)</td>
<td>1.0 (0.6-1.8)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32 (54.2)</td>
<td>2.0 (1.2-3.6)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5 (35.7)</td>
<td>0.8 (0.3-2.5)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>182</td>
<td>37 (58.7)</td>
<td>Ref</td>
<td>27 (45.8)</td>
<td>Ref</td>
<td>9 (64.3)</td>
<td>Ref</td>
</tr>
<tr>
<td>Obtained blood or other specimens from dogs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>137</td>
<td>23 (36.5)</td>
<td>0.7 (0.4-1.2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24 (40.7)</td>
<td>0.8 (0.4-1.4)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5 (35.7)</td>
<td>0.7 (0.2-2.1)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>169</td>
<td>40 (63.5)</td>
<td>Ref</td>
<td>35 (59.3)</td>
<td>Ref</td>
<td>9 (64.3)</td>
<td>Ref</td>
</tr>
<tr>
<td>Wore gloves when exposed to sick dog</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>166</td>
<td>30 (50.9)</td>
<td>0.7 (0.4-1.3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42 (73.7)</td>
<td>2.5 (1.3-4.8)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7 (53.9)</td>
<td>0.9 (0.3-2.6)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Yes</td>
<td>122</td>
<td>29 (49.2)</td>
<td>Ref</td>
<td>15 (26.3)</td>
<td>Ref</td>
<td>6 (46.2)</td>
<td>Ref</td>
</tr>
</tbody>
</table>

Note: MN=Microneutralization assay; NI=Neuraminidase inhibition assay.

<sup>a</sup> Covariate has some missing data.  <sup>b</sup> Proportional odds model used.  <sup>c</sup> Fisher's exact test used.  <sup>d</sup> Wald chi-square test used
Table 14. Risk factors associated with a microneutralization assay titer $\geq 1:10$ for canine influenza virus using multivariate conditional logistic regression for the entire study population.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Unadjusted OR (95% CI)</th>
<th>Saturated Model OR (95% CI)</th>
<th>Final Model OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age group (yrs)$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56-78</td>
<td>3.3 (1.1-9.9)$^b$</td>
<td>2.6 (0.7-9.2)</td>
<td>2.8 (0.8-9.2)</td>
</tr>
<tr>
<td>46-55</td>
<td>8.8 (3.2-24.2)$^b$</td>
<td>10.4 (3.2-33.8)</td>
<td>10.3 (3.4-31.6)</td>
</tr>
<tr>
<td>33-45</td>
<td>8.7 (3.1-24.0)$^b$</td>
<td>8.2 (2.5-26.7)</td>
<td>8.8 (2.8-27.4)</td>
</tr>
<tr>
<td>26-32</td>
<td>0.4 (0.1-2.3)$^b$</td>
<td>0.5 (0.1-2.6)</td>
<td>0.4 (0.1-2.5)</td>
</tr>
<tr>
<td>18-25</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
</tbody>
</table>

| Canine exposure                    |                        |                             |                        |
| Yes                                | 1.8 (0.9-3.3)$^b$      | 1.1 (0.5-2.6)               | --                     |
| No                                 | Ref                    | Ref                         | --                     |

| Ever received a human influenza vaccine$^a$ |                        |                             |                        |
| Yes                                | 2.1 (1.1-3.8)$^c$      | 2.0 (1.0-4.2)               | 2.5 (1.3-4.8)          |
| No                                 | Ref                    | Ref                         | Ref                    |

| Human H3N2 influenza titer$^a$       |                        |                             |                        |
| $\geq 1:40$                         | 1.8 (1.1-3.1)$^c$      | 1.5 (0.8-3.0)               | --                     |
| $<1:40$                             | Ref                    | Ref                         | --                     |

$^a$ Covariate has some missing data.

$^b$ Proportional odds model used.

$^c$ Wald chi-square test used.
Table 15. Risk factors associated with a microneutralization assay titer $\geq 1:10$ for canine influenza virus using multivariate conditional logistic regression for only those exposed to dogs.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Unadjusted OR (95% CI)</th>
<th>Saturated Model OR (95% CI)$^c$</th>
<th>Final Model OR (95% CI)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age group (yrs)$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56-78</td>
<td>2.7 (0.7-10.3)$^b$</td>
<td>2.4 (0.5-12.5)</td>
<td>2.6 (0.5-13.5)</td>
</tr>
<tr>
<td>46-55</td>
<td>6.9 (1.9-24.9)$^b$</td>
<td>10.1 (2.2-47.5)</td>
<td>10.1 (2.1-47.4)</td>
</tr>
<tr>
<td>33-45</td>
<td>7.9 (2.2-28.7)$^b$</td>
<td>10.0 (2.0-48.5)</td>
<td>10.3 (2.1-50.4)</td>
</tr>
<tr>
<td>26-32</td>
<td>0.3 (0-2.6)$^b$</td>
<td>0.3 (0-3.7)</td>
<td>0.3 (0-3.6)</td>
</tr>
<tr>
<td>18-25</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Ever received a human influenza vaccine$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2.1 (1.1-3.9)$^c$</td>
<td>2.1 (0.9-4.6)</td>
<td>2.4 (1.2-4.8)</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Human H3N2 influenza titer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\geq$1:40</td>
<td>1.8 (1.0-3.4)$^c$</td>
<td>1.3 (0.5-2.9)</td>
<td>--</td>
</tr>
<tr>
<td>&lt;$1:40</td>
<td>Ref</td>
<td>Ref</td>
<td>--</td>
</tr>
<tr>
<td>Examined dogs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.6 (0.3-1.0)$^b$</td>
<td>0.3 (0.2-0.7)</td>
<td>0.3 (0.2-0.7)</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Wore PPE when working with dogs$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never/Rarely</td>
<td>0.6 (0.3-1.0)$^b$</td>
<td>0.4 (0.2-0.8)</td>
<td>0.4 (0.2-0.8)</td>
</tr>
<tr>
<td>Sometimes/Most of the time/Always</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
</tbody>
</table>

$^a$ Covariate has some missing data.

$^b$ Proportional odds model used.

$^c$ Wald chi-square method used.
Table 16. Risk factors associated with a positive neuraminidase inhibition assay result for canine influenza virus using multivariate conditional logistic regression for the entire study population.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Unadjusted OR (95% CI)</th>
<th>Saturated Model OR (95% CI)</th>
<th>Final Model OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age group (yrs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56-78</td>
<td><strong>14.1 (5.5-35.8)</strong></td>
<td><strong>12.6 (4.2-38.0)</strong></td>
<td><strong>16.0 (5.7-45.2)</strong></td>
</tr>
<tr>
<td>46-55</td>
<td>2.4 (0.9-6.7)</td>
<td>2.5 (0.8-8.0)</td>
<td>3.0 (1.0-8.9)</td>
</tr>
<tr>
<td>33-45</td>
<td>1.1 (0.3-3.6)</td>
<td>1.2 (0.3-4.4)</td>
<td>1.3 (0.4-4.9)</td>
</tr>
<tr>
<td>26-32</td>
<td>2.0 (0.7-5.6)</td>
<td>1.8 (0.5-5.9)</td>
<td>1.8 (0.6-6.1)</td>
</tr>
<tr>
<td>18-25</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Shelter worker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td><strong>0.4 (0.1-1.1)</strong></td>
<td>0.3 (0.1-1.2)</td>
<td>--</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>Ref</td>
<td>--</td>
</tr>
<tr>
<td>Breeder</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td><strong>1.7 (1.0-2.8)</strong></td>
<td>0.9 (0.4-1.8)</td>
<td>--</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>Ref</td>
<td>--</td>
</tr>
<tr>
<td>Ever received a human influenza vaccine*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td><strong>2.2 (1.2-4.0)</strong></td>
<td>1.9 (0.9-3.7)</td>
<td>1.8 (1.0-3.6)</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Exposed to poultry*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td><strong>2.1 (1.1-3.8)</strong></td>
<td>1.2 (0.5-2.7)</td>
<td>--</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>Ref</td>
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</tr>
<tr>
<td>Exposed to horses*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td><strong>1.9 (1.0-3.7)</strong></td>
<td>1.3 (0.6-3.1)</td>
<td>--</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>Ref</td>
<td>--</td>
</tr>
<tr>
<td>Resident of Florida</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.6 (0.3-1.0)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
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</tr>
<tr>
<td>Resident of Wisconsin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.4 (0.1-1.1)</td>
<td>--</td>
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</tr>
<tr>
<td>No</td>
<td>Ref</td>
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<td>--</td>
</tr>
<tr>
<td>Resident of Illinois</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td><strong>3.6 (1.2-10.5)</strong></td>
<td>3.1 (0.7-13.4)</td>
<td><strong>4.7 (1.3-16.6)</strong></td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Resident of Iowa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td><strong>1.5 (0.9-2.7)</strong></td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

*a Covariate has some missing data.

b Proportional odds model used.  c Wald chi-square test used.
Table 17. Risk factors associated with a positive neuraminidase inhibition assay result for canine influenza virus using multivariate conditional logistic regression for only subjects exposed to sick dogs.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Unadjusted OR (95% CI)</th>
<th>Saturated Model OR (95% CI)</th>
<th>Final Model OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age group (yrs)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56-78</td>
<td><strong>16.1 (4.6-56.4)</strong></td>
<td><strong>16.6 (3.4-82.0)</strong></td>
<td><strong>18.5 (5.1-66.9)</strong></td>
</tr>
<tr>
<td>46-55</td>
<td>2.2 (0.6-8.3)</td>
<td>2.8 (0.5-14.8)</td>
<td>2.3 (0.6-9.0)</td>
</tr>
<tr>
<td>33-45</td>
<td>1.2 (0.3-5.4)</td>
<td>1.5 (0.3-9.5)</td>
<td>1.5 (0.3-6.9)</td>
</tr>
<tr>
<td>26-32</td>
<td>0.8 (0.2-4.2)</td>
<td>1.4 (0.2-9.6)</td>
<td>1.0 (0.2-6.9)</td>
</tr>
<tr>
<td>18-25</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td><strong>Shelter worker</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td><strong>0.3 (0.1-0.9)</strong></td>
<td>0.3 (0.1-1.3)</td>
<td>--</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>Ref</td>
<td>--</td>
</tr>
<tr>
<td><strong>Breeder</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td><strong>1.7 (1.0-3.1)</strong></td>
<td>0.8 (0.3-1.9)</td>
<td>--</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>Ref</td>
<td>--</td>
</tr>
<tr>
<td><strong>Ever received a human influenza vaccine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td><strong>2.6 (1.3-5.2)</strong></td>
<td>1.9 (0.8-4.1)</td>
<td>--</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>Ref</td>
<td>--</td>
</tr>
<tr>
<td><strong>Exposed to poultry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1.6 (0.7-3.6)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td><strong>Exposed to horses</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1.1 (0.5-2.5)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>--</td>
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</tr>
<tr>
<td><strong>Resident of Florida</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.6 (0.3-1.2)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td><strong>Resident of Wisconsin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td><strong>0.2 (0.0-0.9)</strong></td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td><strong>Resident of Illinois</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td><strong>4.5 (1.4-14.3)</strong></td>
<td>3.7 (0.8-17.2)</td>
<td><strong>4.8 (1.3-17.7)</strong></td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td><strong>Resident of Iowa</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1.5 (0.9-2.7)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td><strong>Birthed puppies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td><strong>1.8 (1.0-3.2)</strong></td>
<td>1.4 (0.6-3.2)</td>
<td>--</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>Ref</td>
<td>--</td>
</tr>
<tr>
<td><strong>Wore gloves when exposed to a sick dog</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td><strong>2.5 (1.3-4.8)</strong></td>
<td>1.5 (0.7-3.5)</td>
<td>--</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>Ref</td>
<td>--</td>
</tr>
</tbody>
</table>

* Covariate has some missing data.

* Proportional odds model used.  
* Wald chi-square test used.
Table 18. Risk factors associated with positive neuraminidase inhibition and microneutralization assay results for canine influenza virus using multivariate conditional logistic regression for the entire study population.

<table>
<thead>
<tr>
<th>Risk Factor(^a)</th>
<th>Unadjusted OR (95% CI)</th>
<th>Final Model OR (95% CI)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age group (yrs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>56-78</td>
<td>3.4 (0.3-181)(^b)</td>
<td>4.0 (0.2-248)</td>
</tr>
<tr>
<td>46-55</td>
<td>8.7 (1.1-396)(^b)</td>
<td>12.2 (1.4-585)</td>
</tr>
<tr>
<td>33-45</td>
<td>3.4 (0.3-183)(^b)</td>
<td>4.6 (0.4-249)</td>
</tr>
<tr>
<td>26-32</td>
<td>2.2 (0.1-131)(^b)</td>
<td>3.0 (0.2-181)</td>
</tr>
<tr>
<td>18-25</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Exposed to poultry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.3 (0.1-1.0)(^c)</td>
<td>0.2 (0.1-0.8)</td>
</tr>
<tr>
<td>No</td>
<td>Ref</td>
<td>Ref</td>
</tr>
</tbody>
</table>

\(^a\) Covariates have some missing data.

\(^b\) Fisher's exact method used.

\(^c\) Wald chi-square method used.
Table 19. Serologic results for human antibodies against canine respiratory coronavirus based on optical density (OD) readings between exposed and non-exposed study groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Exposed (n=302)</th>
<th>Controls (n=99)</th>
<th>p-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean OD (SD)</td>
<td>-0.03 (0.11)</td>
<td>-0.03 (0.12)</td>
<td>0.97</td>
<td>1.02 (0.7-1.5)</td>
</tr>
<tr>
<td>OD Quartiles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First</td>
<td>75 (24.8)</td>
<td>24 (24.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second</td>
<td>76 (25.2)</td>
<td>25 (25.3)</td>
<td>0.80</td>
<td>1.1 (0.7-1.6)</td>
</tr>
<tr>
<td>Third</td>
<td>76 (25.2)</td>
<td>25 (25.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fourth</td>
<td>75 (24.8)</td>
<td>25 (25.3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 5
DISCUSSION

Study summary

This study aimed to determine the seroprevalence of three canine diseases among canine-exposed and non-canine exposed human populations, *Brucella canis*, H3N8 canine influenza virus (CIV), and canine respiratory coronavirus (CRCoV), and to identify risk factors associated with evidence of seropositivity. *B. canis* is a known zoonotic disease, but the seroprevalence among humans has not been examined since the 1970s. CIV and CRCoV are emerging canine diseases, for which investigations into potential cross-species transmission have not yet been conducted.

Between 2007 and 2010, a seroepidemiological cohort study was conducted to meet the study aims. A total of 306 canine-exposed participants were enrolled in the study, including veterinary staff, breeders, kennel and shelter employees, racetrack workers, trainers, and dog show enthusiasts. For comparisons, 101 non-canine exposed subjects were enrolled from University of Iowa and University of Florida faculty, staff, and student volunteers. All participants provided a serum sample and completed a self-administered questionnaire. Sera were examined for antibodies against *B. canis*, CIV, and CRCoV. Questionnaire data provided information about canine exposures and possible risk factors for seropositivity. Potential confounders were also assessed.

Antibody responses to infection

Infection occurs when a pathogenic microorganism colonizes and replicates in or on a host. Mounting a humoral immune response against a non-host antigen does not imply infection has occurred within a host. Continued antigen presentation of non-viable pathogens can also stimulate an immune system response (e.g. contaminated dust particles or killed vaccines). Therefore, because this study involved serological sampling at a single time point, study data cannot confirm human infection with these canine
pathogens has occurred. It can only suggest previous exposure to a pathogen for which an antibody response transpired at some time in the past. This exposure may represent antigen exposure without replication in or on the host; however, as recommended by the Centers for Disease Control and Prevention, the magnitude of elevated IgM or IgG antibodies may also be considered as evidence of infection versus simple antigen presentation. Very high IgM or IgG titers often are accepted as evidence of previous infection [118].

Nonetheless, a cohort study of disease seroprevalence is a useful tool to in examining hypotheses regarding the potential of human infection with a novel pathogen. To suggest that a previous infection has occurred, elevated antibodies titers above a defined cut-point can be compared between exposed and non-exposed populations after controlling for potential confounders (e.g. cross-reacting antibodies). If it is found that humans seroconverted in response to an exposure, and specific exposures suggest a possible association between a causative etiological agent and the outcome, future prospective studies may be warranted to determine if the etiological agent is causing infection. An important recent example is the epidemiologic investigations that found evidence of Reston ebolavirus infections in animals spreading to animal workers [123].

During the initial stages of antigen presentation or infection, immunoglobulin M (IgM) antibodies are often produced as a first response to an antigen. Subsequently, IgM falls to levels often undetectable, and IgG antibodies rise. IgG can remain detectable for long periods of time against some antigens. Therefore, elevated IgM antibodies titer often signify a recent infection, whereas detectable IgG antibodies signify a past infection or immunization. For confirmation of an infection (or immunization) via serology, it is imperative to have acute and convalescent sera samples and employ serological assays that distinguish between IgG and IgM antibodies. Serological evidence for a recent infection can be determined if an initial rise in IgM antibody titer is followed by a fall in IgM titer and a subsequent rise in IgG antibody levels (Figure 11) [124]. If a patient is
IgG and IgM negative, this either indicates there was no exposure, or it is too early in the incubation period to detect antibodies. A result of IgG antibody positive but IgM negative would indicate a past exposure to a pathogen. If a patient is positive for both IgG and IgM antibodies, they are most likely experiencing a current or recurrent infection. Lastly, if serological tests indicate a patient is IgG negative but IgM positive, the patient is likely in the early stages of an infection.

However, in instances where the outcome is rare and clinical disease unlikely, to capture acute cases of subclinical illness would be very difficult. Seroprevalence studies are the best epidemiological approach when first examining the potential for rare and seemingly unapparent infections in a population.

**Aim 1: Brucella canis**

This cohort study demonstrated that humans have been exposed to *B. canis* and developed antibodies against the pathogen. Thirteen study subjects had evidence of antibodies against *B. canis* by the rapid slide agglutination test, suggesting they were previously exposed to the bacteria, although some may be false-positive results. Eleven (3.6%) seropositive subjects were canine-exposed, while 2 (1.9%) were non-canine exposed control subjects. Among all study subjects, exposure to a known positive *B. canis* dog was a risk factor for *B. canis* seropositivity (OR=5.9; 95% CI, 1.0-25.5), although this risk lessened when controlling for the demographic characteristic of residing in Iowa (adjusted OR=5.4; 95% CI, 0.9-23.8). Among subjects who reported exposure to whelping dogs, risk factors associated with having antibodies against *B. canis* included exposure to a known *B. canis* positive dog (adjusted OR=7.6; 95% CI, 1.01-48.7) and not wearing PPE when exposed to a whelping dog (adjusted OR=6.0; 95% CI, 1.02-65.0), after controlling for residing in Iowa.

This seroprevalence study expands on the *B. canis* prevalence data collected in the 1970s and suggests that humans continue to be exposed to *B. canis*, with an overall
seroprevalence of 3% among the study population. Because so few seropositives were identified in this enrollment group, the sample size did not provide sufficient power necessary to detect many statistically significant differences between groups for risk factor analyses; with an alpha of 0.05, power equaled 0.12. Nonetheless, data involving those 13 subjects was still valuable. Exposure to a known positive *B. canis* dog was associated with seropositivity, suggesting that the majority of the RSAT positive results truly detected antibodies against *B. canis* and were not false positive results. In addition, not wearing PPE when exposed to a whelping female was also associated with higher likelihood of detectable antibodies against *B. canis*, providing a potential risk factor for human infection with *B. canis*.

The previous literature has identified dog breeders and veterinarians as high-risk occupational groups for *B. canis* infections. While no work/hobby was specifically associated with seropositivity, this may be because subjects were allowed to cite more than one work/hobby. Examination of specific occupations/hobbies of the 11 canine-exposed seropositive subjects revealed previously unconsidered at-risk groups. Two subjects were veterinarians and two were veterinary technicians. While 5 subjects were dog breeders, all 5 reported tending to only 1-3 breeding females, which could be considered small breeding kennels. Of the 89 subjects who reported working in the dog breeding industry or breeding dogs as a hobby, the average number of breeding females to which the subjects tended was 13.2 (ranging from 0 to 300 females). Another subject had spent 4 years as a dog trainer, 2 years as a groomer, and one year as both a kennel employee and shelter worker. Lastly, one subject reported his only canine exposure to be through dog shows for the past 20 years and reported no previous exposure to breeding dogs.

Power calculations and sample size were difficult to estimate *a priori*, as zoonotic transmission of *B. canis* is not well documented. A sample size of 400 with an exposed to non-exposed ratio of 3:1 was selected. As the study prevalence was 3.6% for the
canine-exposed population and 2% for the non-canine exposed population, this sample size was not large enough to detect significant differences between the groups (power = 0.12).

Because exposure to sheep was not associated with seropositivity, it is assumed that cross-reacting antibodies against *B. ovis* did not occur. Results also indicated that exposure to cattle, pigs, goats, deer, and elk was not associated with a positive RSAT result. In addition, all 4 subjects who reported accidental injections with *B. abortus* vaccines, which have been shown to cause human illness [125-128], were negative by the RSAT. These findings suggest that cross-reactivity between other *Brucella* species, including *B. abortus, B. melitensis,* and *B. suis,* did not occur with the 2ME-RSAT. Cross-reactivity was not expected because *B. canis* and *B. ovis* have a rough lipopolysaccharide (LPS), while *B. abortus, B. melitensis,* and *B. suis* have smooth LPS. Rough and smooth antibodies do not cross-react with the other; therefore, cross-reactivity in the 2ME-RSAT with smooth LPS species was not expected [129, 130]. Cross-reactivity between *B. canis* and other gram-negative bacteria can occur in some tests, particularly agglutination tests, but the addition of 2ME should prevent most of this potential cross-reactivity [47].

The cut off qualification for canine exposure was set at 5 years. For *B. canis,* dogs can remain culture positive for years, and seropositivity can last 6-12 months [48]. In the report of the first naturally acquired human case of *B. canis,* the woman sustained a low seropositivity one year after completion of therapy [63]. Persistence of IgG is presumably associated with persistence of clinical disease (seen for 6-11 months), while IgM may remain for years in persons with subclinical infections [105]. In addition, antibodies against the cell wall *B. canis* generally last only during bacteremia, while antibodies against cytoplasmic antigens persist longer (6-12 months) [106]. Long-lasting seropositivity has been observed for *B. abortus* (17 and 45 months after presentation of clinical symptoms), so the same may hold true for *B. canis* [111]. Five years seemed a
sensible midpoint so as to minimize misclassification of exposed as unexposed or vice versa. The most recent cited exposures to *B. canis* positive dogs occurred as far back as 1985 for one of the seropositive subjects, suggesting a not previously reported potential for long-lasting immunity. Although recall bias cannot be excluded, as it may be the subject did not report a more recent exposure, or the subject may have been exposed to an undiagnosed infected dog more recently.

We can only speculate regarding the two control subjects' seropositivity. One subject did cite 9 years of exposure to 2 pet dogs more than 5 years in the past. Perhaps, as suggested from the exposed group, antibodies persist longer than 5 years and the set exposure cut-off of 5 years was not long enough. Interestingly, through personal communication it was revealed that the other subject's father was a veterinarian in a developing country but the subject could not remember any personal dog exposure. Alternatively, these may represent false positive results, for which further confirmation by additional testing is warranted.

The 2ME-RSAT assay was rapid (dependable results in less than 10 minutes), accurate (false negatives are rarely reported), and sensitive (able to detect both acute and chronic stages of infections). Even though the Synbiotics kit employed a suspension of whole *B. ovis* stained with Rose Bengal, based on published works [111], personal consultations, and this study's results, the RSAT canine kit transitioned effectively to human sera to detect human antibodies against *B. canis*. While false negatives are rare, the RSAT may have a high percentage of false positives due to shared antigenic epitopes of lipopolysaccharides of other bacteria [48]. The whole bacteria antigen RSAT is most effective for detecting antibodies against *Brucella* in the early stages of infections (IgM antibodies). Employing the 2ME-RSAT assay variant reduces the potential for cross-reacting IgM antibodies, and thus more accurately measures IgG antibodies which are indicative of a prior infection (IgG may not be present until after 12 weeks of infection). While this scenario is not ideal for diagnosing clinically ill dogs, the 2ME-RSAT
provides a means to detect long-lasting IgG antibodies in humans to indicate a previous Brucella exposure. For this seroprevalence study, a positive 2ME-RSAT result suggests a) a subject was previously exposed to \textit{B. canis} (or \textit{B. ovis}) and mounted an immune response that included long-lasting production of IgG; b) a subject was recently exposed to \textit{B. canis} (or \textit{B. ovis}) and mounted an immune response that included IgM production that was not removed by the addition of 2ME; c) the subject possessed cross-reacting antibodies (most likely IgG) that agglutinated with the RSAT antigen due to a low assay specificity. A negative 2ME-RSAT result suggests a) a subject was not previously exposed to \textit{B. canis} (or \textit{B. ovis}); b) the subject was recently infected for which a humoral antibody response has not yet been mounted or the reacting IgM antibodies were removed with the addition of 2ME (this would result in a positive RSAT screen and secondary negative 2ME-RSAT confirmation); c) the subject had previously been exposed to \textit{B. canis} but a lack of assay sensitivity resulted in a false negative.

Previous literature has suggested that the 2ME-RSAT is a suitable tool for screening human sera for antibodies against \textit{B. canis}; however, these results need to be confirmed with a more specific serological assay, such as an indirect ELISA previously developed which showed a reported 100% specificity and 100% sensitivity [111]. Therefore, the 2ME-RSAT results from this serological study will be confirmed with an indirect ELISA using \textit{B. canis} antigen developed by Dr. Nadia Lucero of the Brucellosis Laboratory at the National Administration of Laboratories and Health Institutes, Buenos Aires, Argentina. In addition, sera will also be tested for antibodies against smooth \textit{Brucella} spp. with the buffered plate antigen test.

Overall, results indicated that persons who share several canine-exposed occupations/hobbies not considered in previous studies, including jobs in small breeding kennels and dog show handlers, may be at increased risk for exposure to zoonotic \textit{B. canis}. Not wearing PPE when caring for a whelping dog proved to increase one's odds of having antibodies against \textit{B. canis}. In addition, survey results indicated 35% of breeders
are not performing *B. canis* testing in their kennels. At-risk populations should regularly test their dogs for *B. canis* and follow standard personal safety precautions to reduce their risk of zoonotic *B. canis* infection.

**Aim 2: H3N8 Canine influenza virus**

Serological data suggested that humans have been exposed to H3N8 CIV and mounted an immune response; however, assay cross-reactivity likely from antibodies against human influenza viruses made it difficult to ascertain accurate seroprevalence data and potential risk factors for seropositivity. Among all 407 study subjects, 76 (18.7%) had elevated antibody titers (>1:10) against CIV by the microneutralization (MN) assay; 63 (20.6%) were among the 306 subjects who reported recent exposure to dogs and 13 (12.9%) were among the 101 subjects who reported no exposure to dogs in the last 5 years. There was no significant difference between the 2 exposure groups (OR=1.7; 85% CI, 0.9-3.3). For the neuraminidase inhibition assay, 75 (18.5%) of the 407 subjects tested either weak or strong positive for CIV antibodies, including 59 (19.4%) canine-exposed subjects and 16 (15.8%) non-exposed subjects (OR=1.3; 95% CI, 0.7-2.4). While the seroprevalence was similar between the 2 assays, the results did not correlate (κ = 0.05 and Pearson's *r* = 0.02). Only 17 (4.2%) subjects were positive by both the MN and NI assays: 14 (4.6%) canine-exposed and 3 (3.0%) non-exposed (OR=1.6; 95% CI, 0.4-8.7). Logistic regression identified associations between older age and prior human influenza vaccinations with CIV seropositivity, in addition to residing in Illinois and contact with poultry. Occupational exposures associated with CIV seropositivity included examining dogs (adjusted OR=0.3; 95% CI, 0.2-0.7) and not wearing PPE when working with dogs (adjusted OR=0.4; 95% CI, 0.2-0.8), although both were protective factors.

When dichotomizing data based on an arbitrary cut-point titer (1:10) and analyzing with binary logistic regression, some data are lost. Discarding data is
especially concerning when the disease is rare or the serological assay is imperfect. The proportional odds model provides superior power to detect risk factors by keeping the data in its original form and using ordinal levels of antibody titer [122]. For the proportional odds assumption, all estimates must be equal across different levels, meaning that the odds of being above any titer cut-point are the same for all titer cut-points. Figure 10 illustrates this assumption, where equal odds ratios can be found at any cut-point; the proportional odds assumption is not violated. For this study population, the proportional odds assumption was met for MN titer results (Figure 5); therefore, proportional odds modeling could be utilized to further examine MN titer and possible risk factors. In addition, proportional odds could be applied to the NI assay, as results were classified in ordinal categories of negative (1), weak positive (5), and strong positive (10), and the proportional odds assumption was also met. Employing the proportional odds model when appropriate improved the overall power of the study.

Because MN and NI results did not correlate ($\kappa = 0.05$ and Pearson's $r = 0.02$), associations between exposures and outcomes were measured for several possible outcomes (elevated MN titer, NI positive result, and both an elevated MN titer and NI weak/strong positive). Upon multivariate conditional logistic regression, no outcome seemed more predictive of true exposure than the other after controlling for potential confounders. All were confounded by older age, as it is likely that older adults have had more time to accumulate influenza vaccines as well as more chances to be infected with human and other zoonotic influenza viruses.

For the MN assay, occupational canine exposures of examining dogs and never/rarely wearing PPE when working with dogs were protective factors for an elevated CIV antibody titer, when controlling for age and prior receipt of human influenza vaccination. These results suggest that occupational canine exposures are playing a role in CIV seropositivity; however, the questionnaire may not have accurately
recorded subjects' behaviors and contact with dogs because of the inverse relationship between exposure and outcome.

NI assay results suggested that the eldest age quartile (56-78yo) had markedly elevated odds of CIV seropositivity compared to the other age categories. When considering prior receipt of human influenza vaccines, the outcome's association with both age and state of residence increased; therefore, influenza vaccination was included in the final multivariate model.

Even though the results of the 2 serological assays did not correlate, statistical analyses were still performed on the 17 subjects who tested positive by both assays, in an attempt to learn more about possible associations between exposures and the outcome. While no occupational canine exposures were associated with dual seropositivity, older age (46-55yo) was associated with a higher likelihood. Conversely, exposure to poultry was associated with a decreased likelihood of dual seropositivity. The outcome of dual seropositivity seemed to be the least robust of the 3 outcomes, based on the statistical results. This indicates either one or both assays were not successful in identifying true exposures to CIV; conversely, as only 17 subjects had dual seropositivity, the small sample size did not provide enough power to detect differences among the subjects. With an alpha of 0.05, the power for the detected seroprevalences between the exposure groups by the MN assay, NI assay, and combined assays was 0.50, 0.16, and 0.11, respectively.

Based upon the serological results for the MN and NI assays, risk factor analyses indicated the assays were confounded by cross-reacting antibodies against human influenza viruses. This was expected more for the MN assay, as it detects HA antibodies; on the contrary, for the drawback to appear with the NI assay was disconcerting as the assay is designed to detect NA antibodies. No human influenza virus with the N8 protein exists; nevertheless, the results of this study suggest that the NI assay is not ideal for detecting unique zoonotic influenza virus infections in humans. The only known cross-neutralization has been documented between NA1 and NA4 specific antibodies; however,
reaction with other NA specific antibodies with the NA8 may be occurring. This may be especially true for human influenza viruses that circulated decades ago, such as the human H2N2 influenza virus, which would explain the association with older age observed in this study population.

The 3 influenza serological assays employed for the study (MN, NI, and HI) detect all serum antibodies that neutralize or recognize influenza viral antigens, including both IgM and IgG; therefore, it is difficult to interpret a positive serological result. Serological evidence for a recent influenza infection can be determined if an initial rise in IgM antibody titer is followed by a fall in IgM titer and a rise in IgG antibody levels [124]. If a subject tests negative, this either indicates there was no previous exposure to the virus, or that it is too early in the incubation period to detect antibodies. Alternatively, the assay sensitivity was poor, for which it could not detect a true positive and falsely identified cases as negative. Because a positive result cannot distinguish between IgG and IgM antibodies, the stage of seroconversion cannot be determined for subjects who test positive. The subject either is in the early stages of infection, is experiencing a current or recurrent infection, or was exposed to the virus sometime in the past. Alternatively, the assay specificity was poor, for which it falsely categorized the subject as positive when in fact no antibodies were present in the sera. This is most often caused by cross-reacting antibodies from previous infection or vaccination with human influenza viruses.

Because of the miscorrelation between MN and NI assay results, an immunodot blot assay employing a recombinant N8 protein was developed. By eliminating the HA protein altogether, potential cross-reactivity with human HA antibodies was controlled; however, various dot lots methods were never successful in detecting antibodies against H3N8 CIV. It is still important to confirm the MN and NI assay results with proper laboratory validations; therefore, a Western blot assay is being developed using whole virus as a possible validative assay.
Overall, even with the evidence of antibody cross-reactivity, the serological results suggested that the canine-exposed population had higher odds for CIV seropositivity, although the odds were not statistically significant. True human exposures to CIV may be occurring at a low level of incidence, for which this sample size was not large enough to detect a significant difference between the exposure groups (power ≤ 0.5). The inverse association between elevated antibodies against CIV with the occupational exposures of examining dogs and wearing PPE when working with dogs is perplexing, but nonetheless suggests contact with dogs is playing a role in the evidence of antibody production against CIV. More research is warranted to further investigate the incidence of CIV infections in man and identify risk factors associated with infection.

Aim 3: Canine respiratory coronavirus

This study aimed to determine if humans had been exposed to the recently discovered canine respiratory coronavirus (CRCoV) and mounted an immune response against the virus. Based on the current published literature, this is the first study to examine the possibility of zoonotic infections with CRCoV among humans. Results show no evidence of previous exposure to CRCoV among persons occupationally exposed to dogs, as no antibodies against CRCoV were detected. There was no difference in ELISA OD between dog workers and unexposed controls.

Because of the antigenic similarities between CRCoV and the human coronavirus OC43, cross-reactivity was a substantial obstacle to overcome when designing a serological assay. A competitive ELISA was developed to control for cross-reacting antibodies and detect specific antibodies against CRCoV. Upon analysis of all subjects, all sera were negative for CRCoV antibodies and the mean optical density ([average of the test wells]-[average of the negative control wells + 3 standard deviations]) between the exposed and non-exposed groups was identical at -0.03.
This study had a number of limitations. Culturing CRCoV proved difficult and was a major obstacle of this study. As previously reported [88], virus was grown on HCT-8 cells. Virus could be propagated at low titers, but total RNA examined from both the infected cells and culture supernatant indicated the canine virus was often trapped inside the human cells and could not readily escape to infect new cells. In an attempt to achieve high virus titers necessary for serological assays, CRCoV was also cultured in 2 canine respiratory tract cell lines provided by Dr. Amy McNeill, University of Illinois, Champaign-Urbana, IL. Both cell lines were successfully infected with CRCoV; however, the titer never surpassed the threshold of that provided by HCT-8 culturing. For further attempts to achieve higher viral titers with HCT-8 cells, the infection and growth conditions were varied and optimized. A suspension infection without trypsin and 2% FBS proved most successful, as well as a lower incubation temperature (35 vs 37°C). In addition, after 5-7 viral passages, the titer would dramatically decrease; therefore, for the competitive ELISA, p1 virus was used.

A significant limitation of examining novel zoonotic transmission of an infectious disease is the lack of proper positive and negative assay controls. There exists no human sera from a known human case of CRCoV. Furthermore, cross-reactivity with antibodies against HCoVs that cause up to 30% of common colds [97] and well as with distantly related enteric HCoVs, makes finding truly non-exposed negative control sera difficult as well. Without these human serum controls, this study employed canine serum as a basis for assay success. While appropriate and essential for this study of a potentially zoonotic disease, this parallel was not ideal as canine serum is intrinsically different to human serum. Most notably was the different concentrations of serum and secondary antibodies required, as human sera contained a much higher level of non-specific binders leading to high background.

CRCoV serological assays (competitive ELISA and IFA) were first developed using human and canine secondary antibodies that detected both IgM and IgG antibodies.
However, this resulted in a high background (most likely attributable to non-specific IgM antibodies), to which a positive result was not discernible compared to a negative result. Due to this high background, the assays were modified to detect only IgG antibodies.

During the initial stages of infection, IgM antibodies are produced as a first response to an antigen. Subsequently, IgM falls to levels often undetectable, and IgG antibodies rise. IgG can remain detectable for long periods of time against some antigens. Detecting IgG antibodies against CRCoV would indicate a past exposure to a pathogen or that the subject is experiencing a current or recurrent infection. An assay detecting IgG alone will not identify a subject in the early stages of an infection. Therefore, the negative results of this study suggest several possible scenarios: 1) no one in the study population has been previously exposed to CRCoV, which could be extrapolated further to suggest that CRCoV has not developed zoonotic capabilities; 2) the assay had low sensitivity that was insufficient in detecting true positives; 3) recent CRCoV exposures had occurred, but the assay did not detect existing IgM antibodies; 4) the assay did not correctly work and no conclusions regarding the study population's exposures can be made. It is possible that low levels of antibodies against CRCoV were present in human sera, but the competition with HCoV OC43 defeated its own purpose, cross-reacted with CRCoV antibodies, and blocked them from detection in the ELISA.

This study supports the premise that humans are not at risk for CRCoV infections; however, infrequent cross-species transmission of CRCoV cannot be ruled out. Cross-reactivity is an inherent limitation for a serological approach to detecting human exposure to CRCoV. Either more sensitive serological techniques using recombinant proteins will need to be developed, or a prospective study designed to detect actual virus shedding during clinical infections may someday be warranted.
Potential limitations

This cohort study of disease seroprevalence faced numerous limitations. The two limitations with the largest implications were the sample population and the inherently imperfect nature of serological assays. While a sample size of 400 with a 3:1 ratio of exposed to non-exposed was appropriate for a pilot study to first explore potential zoonotic transmission of canine diseases, a much larger sample size would be necessary to detect differences between the likely low seroprevalence.

In addition, identifying a truly non-canine exposed control group would be nearly impossible with the popularity of dog ownership. An exposure cut-off of 5 years was selected to achieve the fine balance between enrolling a large control population but limiting the potential of long-lasting antibodies against these canine diseases to confound study results. Because of the seropositivity among the control group, it cannot accurately be determined if antibodies against human pathogens are confounding serological results or if in fact, these subjects mounted a serological response when exposed to dogs more than 5 years ago. Comparisons between the exposure groups would have been more accurate if the canine exposure cut-off was increased to 10-20 years, as well as if lifetime dog exposure data was collected from all study participants.

Sampling bias prevented this study from achieving an objectively represented study population, particularly for the canine-exposed group. Subjects were enrolled via a convenience sampling approach, for which the primary recruitment method was an introductory letter mailed to potential participants with a telephone call follow-up. Not all potential participants could be reached via phone calls, and refusals to participate were common. It can be assumed that inherent differences existed between respondents and non-respondents (Table 20). Particularly of concern is that enrollments most often occurred at participants' home or place of employment, so as to make participation convenient for the subjects. Those who refused to participate, such as greyhound racetracks and dog breeders, may have refused out of concern for having unwanted
visitors on their property. It is feasible that those who refused to participate did not have kennels as sanitary as those who agreed to have study personnel visit their property; therefore, this sampling bias may have excluded those individuals who would most likely have diseased dogs and thus have the highest risk for zoonotic infections. The sample population included in this study most likely included willing participants who had clean facilities and felt they had nothing to hide from outsiders. This sampling bias ultimately affected the external validity of the study, meaning that the risks identified for this study population could not be generalized to all people occupationally exposed to dogs.

The second key limiting factor was the inherently imperfect nature of serological assays. By design, antibodies are not rigidly specific for good reason. Infection with one virus or bacteria can render a person immune to attack by a closely-related pathogen, thus reducing the incidence of infections. In nature, this is a good thing. In serological diagnoses, this is an obstacle difficult to overcome. Because completely controlling for cross-reacting antibodies is often unachievable, epidemiological studies frequently employ comparison groups and statistical adjustments to control for this limitation. In the case of this study, both of these approaches did not overcome the lack of antibody specificity seen for these canine pathogens, in particular CIV and CRCoV. Either a larger sample size or more sensitive serological assays are necessary to accurately identify human exposure to CIV and CRCoV.

In addition to the sample population and serological cross-reactivity, the lack of prevalence data for *B. canis*, CIV, and CRCoV among dogs in the study areas weakened the strength of this study. Without prevalence data of these diseases in the canine population, it is difficult to distinguish if negative results indicated the pathogens were not zoonotic or if people were not being exposed to them in the first place. The limited resources afforded to this study did not permit simultaneous sampling of the dogs cared for by the study participants.
Enrolling 306 canine-exposed subjects from a range of occupations provided the opportunity to examine several professions not previously considered at risk for exposure to canine diseases; however, the generation of multiple subgroups made conducting statistical analyses between these smaller groups much more difficult and obtaining statistical significance impossible. Even more, participants were allowed to cite more than one exposure on the questionnaire. While this enabled a wide spectrum of data to be collected, the categories were not mutually exclusive, and an individual's exposures by each occupation could not be deciphered. Because of the small number of *B. canis* seropositive subjects, analyses of individual exposures could be examined; this was not as practicable for the 151 subjects seropositive for antibodies against CIV.

Lastly, as most evident by the risk factor analysis for CIV (inversely correlated), questionnaire data may not have accurately measured occupational exposures to ascertain potential risk factors associated with elevated antibody titers. Upon reflection, certain questions could have been better worded such that subjects had a clearer understanding of what was being asked. For example, question #9 that asked about lifetime contact with a variety of animals was difficult to interpret for many of the participants and a verbal explanation by the interviewer was often necessary. A fine balance is required between asking every question in which the investigator is interested and presenting a concise questionnaire that avoids interviewee fatigue. While the length of the questionnaire was acceptable, some of the questions were not used in statistical analyses, and additional questions that better ascertained occupational exposure could have been included. A more detailed frequency scheme could have been presented, rather than the often used "never, rarely, sometimes, most of the time, and always". Further details about personnel protective equipment were warranted in order to better understand in what setting workers chose to wear PPE and when they chose forego protection. Also, ascertaining exposures for each specific occupation would have been valuable, rather than asking about canine exposures in broad terms.
**Strengths**

While seroepidemiological studies have their limitations, they are often employed as a valid first step in examining the potential for zoonotic spread of these pathogens. In spite of the aforementioned study limitations, this study's novelty made its implementation worthwhile. While *B. canis* is already recognized as the etiological agent of infections in humans and dogs, no studies since the 1970s have examined its seroprevalence among human populations. To the authors' knowledge, this was the first study to ever examine whether humans had previously been exposed to CIV and CRCoV. Historically, other influenza and corona viruses have developed the ability to cross-species barriers, which carried serious consequences (e.g. SARS and the recent H1N1 swine influenza pandemic). Hence, there were valid public health reasons to conduct this study in spite of the aforementioned study limitations.

In addition, the data collected through this study, including sera and questionnaire data can be banked for future studies, including testing with more robust serological assays, as a baseline serological result compared to follow-up sampling in the future, or to examine for evidence of antibodies against other pathogens.

**Future studies**

This study aimed to determine the seroprevalence of three canine diseases, *B. canis*, H3N8 CIV, and CRCoV, among canine-exposed and non-canine exposed human populations, and to identify risk factors associated with evidence of seropositivity. The study was designed as a cohort study of disease seroprevalence with a one-time serum collection. The cross-sectional nature of this design prevented inferences from being made regarding causation between the suspected etiologic agents and human infections. Because this seroprevalence study suggested evidence of previous exposure to *B. canis* and CIV among the study population, future studies to further understand the zoonotic potential of these pathogens is warranted. In addition, the inconclusive results for
CRCoV also warrant further investigation because potential human exposures to CRCoV were not excluded.

The target population used for this study was satisfactory for capturing a variety of canine-exposed occupations with an assortment of potential dog exposures; however, as realized from this current study, the sample size needs to be increased to account for the expected low seroprevalence among the study population. A study population of 2000 or more would be most ideal (Table 2). In addition, a sounder non-canine exposed control group for comparisons would be desirable, for which previous canine exposures are more limited and better documented.

To broaden the scope of a serological study and develop a stronger argument for disease etiology, a prospective cohort study that employs a systematic sampling scheme is most ideal as a future research direction. The study design could involve further serological investigations that include multiple sera collections. By collecting sera at varying time points, a change in antibody titer levels can be examined, as is the case when comparing acute and convalescent sera samples following exposure to an infectious agent. This approach would be most effective when clinical illnesses can be identified, with acute sera samples collected at illness onset and convalescent samples collected 60 days later. Because clinical disease is not expected to occur often, the prospective study could employ monthly sera collections to monitor developing and waning antibodies associated with subclinical illnesses, although conducting serological assays on such a high volume of samples would require a vast amount of time and resources.

In addition to serum collections, routine sampling could provide a means in which to isolate the pathogen from a person. For detecting CIV and CRCoV, respiratory swab samples (e.g. oropharyngeal or nasopharyngeal) could be collected to isolate virus harbored in the respiratory tract. For *B. canis* which elicits intermittent bacteremia, blood samples could be tested for bacteria by blood culture. Again, when examining evidence
of subclinical infections, this method would be most effective if samples were collected on a weekly, or even monthly basis and compared to serological evidence of exposure.

In conjunction with the routine sampling, subjects could also be monitored for a rare clinical illness associated with pathogen exposure. If a subject experiences an influenza-like illness (fever with cough and/or sore throat) during follow-up, they would be instructed to provide respiratory swab samples along with paired acute and convalescent (60 days) sera. While this method would result in a large number of non-specific illnesses, capturing a clinical case of *B. canis*, CIV, or CRCoV would provide further evidence that zoonotic infections can occur.

Along with prospective monitoring of a large human cohort with routine blood and swab collections, a key additional component for this study would be to simultaneously monitor the dogs for which the study participants provide care. This would provide valuable additional data, as negative results among the human population are difficult to discern without comparable prevalence data among the dogs for which the subjects provide care. Most ideally, up to 10 dogs for each subject would be sampled simultaneously to each human specimen collection, in addition to collaborating with their veterinarian to be notified when acute illnesses occur. Knowing the prevalence of these canine pathogens among the dog population allows for a better understanding of the risk level among the human population. For example, if all dogs continually test negative for CRCoV, then the potential for detecting CRCoV exposure among the human subjects would be very low. Alternatively, if a certain subset of dogs were continually testing positive (e.g. large kennel dogs or show dogs), then more targeted human surveillance efforts among those occupations would be warranted. Even more, if an outbreak of one of these canine pathogens is discovered in the study dogs, then an outbreak investigation would simultaneously be conducted for which all people who come in contact with the dogs routinely provide blood and respiratory swab samples for 60 days to monitor for seroconversion and pathogen shedding.
Questionnaire data would be also collected each time biological samples are collected. Questions would ascertain recent occupational and dog exposures to identify potential risk factors for infection. Samples associated with clinical illnesses would capture illness signs and symptoms. A non-canine exposure control population would be enrolled to serve as a comparison group in statistical analyses.

While the above proposed study design depicts the best-case scenario with unlimited resources, executing a study of this magnitude is unlikely. A more realistic next step based on the results of this seroprevalence study is to maintain a similar sample size, prospectively collect blood and respiratory samples every 3 months, and test 1-2 dogs associated with each subject at the beginning of the study. In addition, a defined outbreak among dogs cared for by the subjects would trigger an zoonotic transmission investigation as described above.

Recommendations

For many canine diseases, the most cost-effective and safest approach to controlling zoonotic transmission is to first reduce the incidence of infection in the dogs. Regarding *B. canis*, more universal testing requirements for breeding dogs should be implemented. This study indicated that 35% of dog breeders are not regularly performing *B. canis* testing, nor when acquiring new dogs, using dogs for breeding, or when selling dogs. When considering recall bias, that percentage is likely to be higher as not all breeders will admit to not testing in their kennel. At the very least, as suggested previously [35], all dogs should be tested when entering a kennel and then tested yearly and every time before mating. Because all breeders with 3 or more breeding females are required to have a USDA breeding license, federal mandates should require proof that licensed breeders are testing for *B. canis* in these instances. For controlling the spread of influenza virus, shelter dogs, greyhound racing dogs, and dogs who regularly visit dog parks and boarding kennels should not only have the kennel cough vaccine (for
Bordetella), but to also have the newly released H3N8 CIV vaccine. In addition, transmission of respiratory diseases such as CIV and CRCoV among dogs can be controlled in kennel situations by limiting the number of dogs housed together in one kennel stall, ensuring all dogs are current on all recommended vaccinations, keeping the facility clean and properly maintained, and providing all necessary veterinary care.

This study also identified several occupations/hobbies not previously considered at increased risk for exposure to canine pathogens, including small-scale dog breeders and dog show handlers. When considering preventing the zoonotic spread of these pathogens to humans, physicians and veterinarians should work together to educate people of their potential risks, such that they can take the proper precautions, including wearing gloves when exposed to a whelping dog and always washing their hands after caring for a sick dog. These practical and commonsense safeguards are often overlooked. In addition, physicians may not always consider zoonotic infections in medical differential diagnoses, so they should be encouraged to give special consideration of zoonotic infections for at-risk populations, especially in the case of B. canis infections. Lastly, in order to more accurately detect antibodies against novel pathogens, more sensitive and specific serological assays need to be developed.
Figure 10. Schematic illustration of the proportional odds assumptions using serological response levels as example.

Table 20. Response outcomes associated with the follow-up telephone calls placed after potential subjects were mailed study letters.

<table>
<thead>
<tr>
<th>Occupation</th>
<th>Enrolled</th>
<th>Refused to participate</th>
<th>Reached but did not receive agreement or refusal</th>
<th>Could not be reached</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veterinary clinics (n=80)</td>
<td>12 (15%)</td>
<td>19 (24%)</td>
<td>27 (34%)</td>
<td>22 (28%)</td>
</tr>
<tr>
<td>Shelters (n=17)</td>
<td>4 (24%)</td>
<td>8 (47%)</td>
<td>3 (17%)</td>
<td>2 (12%)</td>
</tr>
<tr>
<td>Breeders (n=28)</td>
<td>5 (18%)</td>
<td>14 (50%)</td>
<td>3 (11%)</td>
<td>6 (21%)</td>
</tr>
<tr>
<td>Kennels (n=51)</td>
<td>14 (27%)</td>
<td>15 (29%)</td>
<td>9 (18%)</td>
<td>13 (25%)</td>
</tr>
<tr>
<td>Pet stores (n=4)</td>
<td>1 (25%)</td>
<td>1 (25%)</td>
<td>2 (50%)</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: This table includes only a subset of the entire target population. It describes the response outcomes for only the potential participants that were mailed invitation letters and then contacted via a telephone call. Participants were also recruited through face-to-face interactions at dog shows, trade shows, greyhound racetracks, and University campuses, for which response outcomes were not monitored.
Figure 11. Levels of virus and antibody during an acute viral infection.

APPENDIX A
ENROLLMENT QUESTIONNAIRE

Canine Zoonoses Study
Enrollment Questionnaire

Today's date

Last Name
First Name

Address

City
State
Zip Code

1. Gender:  ○ Male  ○ Female

2. Date of birth

3. What is the highest level of education you have attained?
   ○ No School  ○ 4 year college degree
   ○ Primary (grades 1-6)  ○ Post-graduate education (Masters, Doctorate)
   ○ Secondary (grades 7-9)  ○ Professional school
   ○ Tertiary (grades 10-12)  ○ Unknown
   ○ 2 year college degree
Canine Zoonoses Study
Enrollment Questionnaire

MEDICAL HISTORY

4. Do you have any chronic medical problems that weaken the immune system? Such as diabetes, kidney disease, cancer, or a blood disease?
   ○ Yes
   ○ No
   ○ Unknown

5. Do you take medications that weaken the immune system? Such as anti-cancer drugs, corticosteroids like prednisone, or other drugs that weaken the immune system?
   ○ Yes
   ○ No
   ○ Unknown

6. Do you currently use or have you ever used tobacco products? Such as cigarettes, cigars, or chewing tobacco?
   ○ Yes (specify)
   ○ No
   ○ Unknown

7. Have you ever received a vaccination for human influenza (flu shot or Flu Mist nasal spray)?
   ○ Yes (specify)
   ○ No
   ○ Unknown

   For which winter seasons did you receive a flu vaccine?
   ○ 2008-09
   ○ 2005-06
   ○ 2007-08
   ○ 2004-05
   ○ 2008-07
   ○ 2003-04

8. Have you ever been accidentally stuck with a needle containing a vaccine for animals?
   ○ Yes (specify)
   ○ No
   ○ Unknown

   Year

   What was the vaccine?

   What animal were you intending to vaccinate?

Page 2
Canine Zoonoses Study
Enrollment Questionnaire

GENERAL EXPOSURE HISTORY

9. Have you ever touched or had close contact (~3 feet) in your lifetime with any of the animals listed below?

<table>
<thead>
<tr>
<th>Animal</th>
<th>No</th>
<th>Unk</th>
<th>Yes &lt;1yr</th>
<th>Yes 1yr+</th>
<th>Total years of exposure</th>
<th>Avg # of animals per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poultry</td>
<td></td>
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<tr>
<td>Cattle</td>
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<tr>
<td>Sheep</td>
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<tr>
<td>Pigs</td>
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<tr>
<td>Goats</td>
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<td></td>
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<tr>
<td>Horses</td>
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<td></td>
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<tr>
<td>Deer/fowl</td>
<td></td>
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<tr>
<td>Pet dogs in your household</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pet cats in your household</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other:</td>
<td></td>
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<tr>
<td>Other:</td>
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</table>

OCCUPATIONAL EXPOSURES

10. Have you ever had close contact (~3 ft) with multiple dogs as part of your work or hobby? Examples of close contact include examining or treating dogs, vaccinating dogs, cleaning up dog waste or dog cages, birthing dogs, etc.

- Yes (specify)
- No (go to Q16, page 7)
- Unknown (go to Q18, page 7)

When was the most recent contact?
- In the last year
- 1 year ago (continue to Q11)
- 2-3 years ago
- 4-5 years ago
- More than 5 years ago (go to Q16, page 7)
Canine Zoonoses Study
Enrollment Questionnaire

OCCUPATIONAL EXPOSURES

11. What type of work/hobby have you done for which you have had close contact (~3 feet) with dogs?

<table>
<thead>
<tr>
<th>Occupation</th>
<th># of years</th>
<th>Hours per day</th>
<th>Avg # of dogs per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breeder</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kennel owner</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kennel employee</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veterinarian</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veterinary staff</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shelter worker/volunteer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Race dog owner</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Racetrack worker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Groomer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trainer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- None  - Unknown

12. What type of work do you perform with dogs? Please mark all that apply:

- Examining dogs
- Birthing puppies
- Treating ill dogs
- Obtaining blood or other specimens from dogs
- Vaccinating dogs
- Other: __________________________

- Cleaning and disinfecting equipment/areas exposed to dogs

13. When working with dogs, how often do you wear any sort of personal protective equipment? Such as gloves, eye protection, surgical mask, etc.

- Never
- Most of the time
- Rarely
- Always
- Sometimes
- Not sure
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**OCCUPATIONAL EXPOSURES**

14. Please indicate with which of the following dogs you have been in close contact (~3 feet) in the last 5 years.

<table>
<thead>
<tr>
<th>MALE DOGS</th>
<th>Yes</th>
<th>No</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actively breeding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-neutered, but non-breeding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bred and later neutered</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutered and never bred</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>FEMALE DOGS</th>
<th>Yes</th>
<th>No</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whelping (in labor)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actively breeding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-spayed, but non-breeding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bred and later spayed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spayed and never bred</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

15. Have you ever been exposed to a whelping dog (i.e., dog giving birth)?
   - Yes (specify)
   - No (go to question 16)
   - Unknown

15a. How many whelping litters are you exposed to in a year?
   - 1-2
   - 3-4
   - 5-8
   - >8
   - Unknown

15b. When caring for a whelping female, do you typically wear any of the following protective clothing? Mark all that apply.
   - Gloves
   - Mask
   - Outer garments
   - Eye protection
   - Protective footwear
   - None

15c. After caring for a whelping female, do you typically wash your hands?
   - Never
   - Rarely
   - Sometimes
   - Most of the time
   - Always
   - Not sure

15d. Do you disinfect whelping areas after cleaning?
   - Yes
   - No
   - Unknown
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OCCUPATIONAL EXPOSURES

16. Have you ever used frozen colostrum when puppies couldn't nurse?
   ○ Yes (specify)
   ○ No
   ○ Unknown

16a. To how many litters have you given frozen colostrum in the last five years?
   ○ 1
   ○ 2
   ○ 3
   ○ 4
   ○ 5
   ○ >5
   ○ Unknown

17. Do you work in the dog breeding industry or breed dogs as a hobby?
   ○ Yes (specify)
   ○ No (go to question 18)
   ○ Unknown

17a. On average, how many breeding female dogs do you tend to?  

17b. In what instances do you, or your employers, for which you work, currently require Brucella canis testing? Please mark all that apply.
   ○ Acquiring new dogs
   ○ Using dogs for breeding
   ○ Selling dogs
   ○ Perform regular testing
   ○ Other: ___________________________
   ○ None
   ○ Unknown

17c. What type of flooring are your dogs housed on the most? (Mark only one)
   ○ Cement
   ○ Grass
   ○ Dirt
   ○ Straw
   ○ Wire
   ○ Other: ___________________________
Canine Zoonoses Study
Enrollment Questionnaire

ILLNESS HISTORY

18. Have you ever been exposed to a sick dog?
   ○ Yes (continue to Q19)
   ○ No (STOP HERE)
   ○ Unknown (STOP HERE)

19. When caring for a sick dog, do you typically wear any of the following protective clothing?
   Please mark all that apply:
   ○ Gloves
   ○ Mask
   ○ Outer garments
   ○ Eye protection
   ○ Protective footwear
   ○ None

20. After caring for a sick dog, how often do you typically wash your hands?
   ○ Never
   ○ Rarely
   ○ Sometimes
   ○ Most of the time
   ○ Always
   ○ Not sure

21. Have you ever become ill after having close contact (~3 feet) with a sick dog?
   ○ Yes (specify)
   ○ No
   ○ Unknown

   What symptoms did you experience?
   ○ Fever
   ○ Loss of appetite
   ○ Cough
   ○ Weight loss
   ○ Sore throat
   ○ Back pain
   ○ Headache
   ○ Arthritis
   ○ Muscle aches
   ○ Depression
   ○ Sweets
   ○ One or more enlarged lymph nodes
   ○ Undue fatigue
   ○ Other:

22. Have any of the sick dogs ever been diagnosed with brucellosis (Brucella canis)?
   ○ Yes (specify)
   ○ No (go to question 23)
   ○ Unknown

   (go to question 23b)

22a. When was your most recent exposure to an infected dog?

   Y M D Y Y Y

22b. Was the Brucella infection associated with a kennel outbreak?
   ○ Yes (specify)
   ○ No
   ○ Unknown

   If yes, how many dogs were affected?
Canine Zoonoses Study
Enrollment Questionnaire

ILLNESS HISTORY

23. Have any of the sick dogs ever been diagnosed with canine influenza virus (CIV)?
   ○ Yes (specify)
   ○ No (go to question 24)
   ○ Unknown (go to question 24)

23a. When was your most recent exposure to an infected dog?
   [ ] [ ] [ ] [ ] [ ] [ ]

   M   M   D   D   Y   Y   Y

23b. Was the influenza infection associated with a CIV outbreak?

   Yes (specify)
   No
   Unknown

   Was the outbreak at an animal race track?
   ○ No
   ○ Dog track
   ○ Dog and horse track

   How many dogs were infected (tested positive)? [ ] [ ] [ ]

24. Have any of the sick dogs ever been diagnosed with kennel cough (canine infectious respiratory disease complex, or CIRD)?
   ○ Yes (specify)
   ○ No (STOP HERE)
   ○ Unknown (STOP HERE)

24a. When was your most recent exposure to an infected dog?
   [ ] [ ] [ ] [ ] [ ] [ ]

   M   M   D   D   Y   Y   Y

24b. Was the kennel cough associated with an outbreak?

   Yes (specify)
   No
   Unknown

   If yes, how many dogs were affected? [ ] [ ] [ ]

24c. Had the dog(s) been vaccinated for “kennel cough”?
   ○ Yes
   ○ No
   ○ Unknown

24d. Were the dogs ever diagnosed with any of these pathogens? Mark all that apply.
   ○ Respiratory coronavirus
   ○ Bordetella bronchiseptica
   ○ Distemper virus
   ○ Herpesvirus
   ○ Adenovirus
   ○ Mycoplasma spp.
   ○ Parainfluenza virus
   ○ Other: ____________________________

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FOR INVESTIGATOR USE ONLY

Was enrollment form reviewed?  ○ Yes  ○ No
Was blood sample obtained?    ○ Yes  ○ No
Was participant given a t-shirt?  ○ Yes  ○ No

I certify that the information recorded here is complete and has been accurately recorded.

Initials     Date

Thank you for participating in this study!
REFERENCES


